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Mosquito-targeted malaria control: alternative uses for existing insecticides and antimalarial drugs

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MOSQUITO-TARGETED MALARIA CONTROL: ALTERNATIVE USES FOR EXISTING INSECTICIDES AND ANTIMALARIAL DRUGS

by

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DEDICATION

Dedicated to all my friends and family who supported me through this journey. Thanks for all the late-night video calls.

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ABSTRACT

Introduction: Malaria is a deadly infectious disease that affects millions of people around the world. This vector-borne tropical disease is caused by parasites that belong to the *Plasmodium* genus and is transmitted through the bite of an infected female *Anopheles* mosquito. Tools such as long-lasting insecticide nets (LLINs) and attractive toxic sugar baits (ATSBs) have been shown to work well in delivering insecticides to mosquitoes, but due to the spread of insecticide resistance throughout sub-Saharan Africa and Southeast Asia, there has been much interest in finding alternative chemical compounds against *Anopheles* mosquitoes. Compounds, such as methoxyfenozide (MET), have been shown to have insecticidal effects against *Anopheles gambiae* females. MET alongside another insecticide, hydramethylnon (HYD), have been shown to have antimalarial properties against mosquito-stage *Plasmodium falciparum* parasites; HYD's insecticidal effects on *A. gambiae* is unknown. Atovaquone (ATQ), a known antimalarial drug for humans, has been observed to have antimalarial properties against mosquito-stage *P. falciparum*. The primary focus of my study is to assess the potential of the three compounds when used in tarsal contact assays (MET) or sugar feeding assays (ATQ, HYD, and MET).

Methods: *A. gambiae* females were exposed to ATQ, HYD, or MET through sugar feeding assays, and MET through tarsal contact assays to assess the effect of these compounds on mosquito survival, *P. falciparum* infection, or mosquito egg development. Effects on *P. falciparum* infection were assessed through oocyst intensity, the prevalence of infection, the oocyst size, and the number of sporozoites present in the salivary glands.

Results: Through sugar feeding, ATQ induced a significant reduction in *P. falciparum* prevalence of infection when *A. gambiae* were exposed to the treated sugars before an infectious blood meal. ATQ had also significant effects on *P. falciparum* oocyst size and number of sporozoites when *A. gambiae* were continuously exposed to the treated sugars after an infectious blood meal. There was a dose-dependent survival effect caused by HYD on *A. gambiae* females through sugar feeding, and also a significant effect of HYD-treated sugar feeding on the prevalence of *P. falciparum* infections. MET had little effect through tarsal contact on infection and egg development but showed significant effects on egg development through sugar feeding.

Conclusion: This study identified important compounds that could be used in ATSBs and LLINs. In particular, ATQ had antimalarial effects when infected mosquitoes were exposed to ATQ-treated sugar meals, and HYD-treated sugar meals had insecticidal effects against *A. gambiae* mosquitoes, with promising implications for the future of vector control and malaria eradication.

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LIST OF ABBREVIATIONS

20E 20-hydroxyecdysone

ACE ..... Acequinocyl

ACT ..... Artemisinin Combination Therapy

A. gambiae ..... *Anopheles gambiae*

ATP ..... Adenosine Triphosphate

ATQ ..... Atovaquone

ATSBs ..... Attractive Toxic Sugar Baits

DBH ..... Dibenzoylhydrazines

DDT ..... Dichlorodiphenyltrichloroethane

DHOD ..... Dihydroorotate Dehydrogenase

DMSO ..... Dimethyl Sulfoxide

DNA ..... Deoxyribose Nucleic Acid

EC50 ..... Half Maximal Effective Concentration

EcR ..... Ecdysone Receptor

EIA ..... Enzyme Immunoassay

ELISA ..... Enzyme-linked Immunosorbent Assay

FL ..... Florida

GDP ..... Gross Domestic Product

GMEP ..... Global Malaria Eradication Program

HEPA ..... High Efficiency Particulate Air

HEPES ..... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HYD ..... Hydramethylnon

ITNs ..... Insecticide-Treated Nets

IRS ..... Indoor Residual Spray

LLINs ..... Long-lasting Insecticide-treated Bed Nets

MA ..... Massachusetts

MET ..... Methoxyfenozide

MI ..... Michigan

NADH ..... Nicotinamide Adenine Dinucleotide

NGO ..... Non-Government Organization

pBM ..... Post-Blood Meal

PBO ..... Piperonyl Butoxide

PBS ..... Phosphate Buffered Saline

P. falciparum ..... *Plasmodium falciparum*

pIBM ..... Post-Infectious Blood Meal

RBC ..... Red Blood Cell

RME ..... Rapeseed Methyl Ester

RPMI ..... Rose Park Memorial Institute

TN ..... Tennessee

UK ..... United Kingdom

VA ..... Virginia

WHO ..... World Health Organization

INTRODUCTION

Malaria is a deadly infectious disease that affects millions of people around the world. This vector-borne tropical disease is caused by parasites that belong to the *Plasmodium* genus and is transmitted through the bite of an infected female *Anopheles* mosquito (WHO, 2019b). Indicators of malaria can be flu-like symptoms, such as high fevers and chills; however, malaria can lead to severe illnesses, such as severe anemia, jaundice, or cerebral malaria. Untreated, malaria can lead to death (Milner, 2018). According to the World Malaria Report 2019, in 2018, there were an estimated 228 million cases worldwide, and out of these cases, there were an estimated 405,000 deaths (WHO, 2019b). With millions of people infected with this parasite and billions of people at risk of infection, malaria eradication should be a priority for health organizations, research institutes, and governments around the world.

I. Burden of Malaria: A Public Health Concern

Malaria is a debilitating illness that not only affects millions of people but also places a huge burden on the communities and countries where malaria is endemic. Eighty-five percent of the burden falls on nineteen countries—of which eighteen countries are in the sub-Saharan Africa region, with the only country outside of this region being India. Ninety-three percent of the malaria cases occurring in 2018 were in the Africa region (WHO, 2019b). In malaria-endemic countries, the disease is one of the factors contributing to an obstinate and complex cycle of poverty. Malaria is referred to

as “a tropical disease of poor countries,” as the countries still battling malaria are some of the poorest countries in the world (Teklehaimanot & Mejia, 2008). In fact, around 58% of the cases worldwide befall the poorest 20% of the world’s population (Breman et al., 2004). While the climate and environmental characteristics of these countries are the main contributors to malaria transmission, those living in poverty are hit hardest due to the cost of preventative measures and treatment (European Alliance Against Malaria, 2007). The insufficient healthcare infrastructures in some malaria-afflicted countries make eradication programs difficult to implement and patients difficult to access for treatment (Nájera et al., 2011). In 2018, 67% of the estimated 405,000 deaths caused by malaria were children under five years, a population that is most vulnerable to malaria infections. These cases remain largely due to the inaccessibility of health care for some families, where as much as 40% of febrile children in sub-Saharan Africa did not have a trained medical provider to treat their symptoms — to put this “40%” into perspective, 24 million children in Sub-Saharan Africa are estimated to be infected with *Plasmodium falciparum* in 2018 (WHO, 2019b). The absence of resources to seek timely treatment, the inability to afford simple prevention measures, and a lack of education and knowledge on malaria prevention and symptoms operate to perpetuate both illness and poverty in these communities (Nájera et al., 2011; Teklehaimanot & Mejia, 2008). In fact, the WHO states that reducing malaria by 10% will increase the annual GDP of a country by 0.3% (WHO, 2019b). With malaria and poverty being so strongly connected, governments, NGOs, and researchers should invest more research and public health effort

and financial support to find better, more affordable, and more accessible tools and treatments for malaria control.

While at present malaria is localized in certain regions of the world, like Africa and Southeast Asia, a century ago the disease was found even in temperate regions such as North America and Europe (Ashley et al., 2018; Talapko et al., 2019). The establishment of the World Health Organization by the United Nations in the mid-1900s put greater priority on eradicating malaria and other infectious diseases worldwide. Eradication efforts, in the form of the WHO's Global Malaria Eradication Program (GMEP), successfully eliminated malaria from or controlled malaria in several regions around the world, including parts of southern Europe, Singapore, Hong Kong, and parts of Malaysia, India, and Sri Lanka, since its establishment in 1955 until the end of the program in 1968 (Majori, 1999; Nájera et al., 2011). Strategy-wise, the program heavily relied on the use of dichloro-diphenyl-trichloroethane (DDT), a commonly used agricultural insecticide from the mid 1900s, and on the use of mass drug administration of chloroquine, an effective antimalarial treatment and prophylaxis (Meibalan & Marti, 2017). However, heavy resistance developed against both DDT and chloroquine, leading to their decreased effectiveness in malaria eradication. Additionally, the GMEP used a "one-size-fits-all" method for malaria eradication worldwide; this approach meant that they were unable to adapt to the greater presence of malaria transmission in less developed regions nor to the increasing resistance against both insecticide and drug (Bleakley, 2010; WHO, 2014). Alongside these failures of GMEP, the decreasing momentum, the lack of continuous eradication efforts, and the decline in funding and

investments in malaria eradication have all contributed to the devastating resurgence of malaria in several countries, many of which were close to elimination in the past. Many tropical countries, where the climate and environment provide the optimal grounds for transmission, are still struggling to end malaria in 2020 (Dhiman, 2019; Nájera et al., 2011). Despite the decreased momentum in malaria eradication after GMEP, renewed interest has brought tremendous improvements and progress in the WHO goals for malaria eradication at the turn of the century.

Progress has been made in several countries. In Figure 1, many countries have shown a significant reduction in malaria cases per year, having zero cases for 3 consecutive years in 2018, when in 2000 they were endemic. Nonetheless, many countries are still afflicted by malaria, with more than one indigenous case a year as highlighted in red (Figure 1). The incidence rate of clinical malaria has decreased by 40% between 2000 and 2015 (Bhatt et al., 2015). Nonetheless, recent data shows that the decreased incidence rate has plateaued between 2014 and 2018 when the incidence rate of malaria has remained 57 cases per 1000 population at risk (WHO, 2019b). This suggests that current methods of intervention and treatment are no longer sufficient in reducing malaria burden. In 2011, a paper in *Cell Press* by Ranson, et al., raised awareness that no new insecticide classes for widespread public health application have been discovered in more than 30 years prior to 2011 due to a lack of research funding, a fact which shows the prior loss of momentum in malaria eradication efforts (Ranson et al., 2011). As mentioned in Nájera's 2011 paper, Mark Boyd, a previous president of the American Academy of Tropical Medicine and the American Society of Tropical

Medicine, described malaria control as not a campaign but a long-term program that should be consistently maintained rather than sporadically administered (Nájera et al., 2011). In line with Boyd's vision of malaria control, it is important to maintain momentum and to continue funding or investing in resources for malaria eradication.

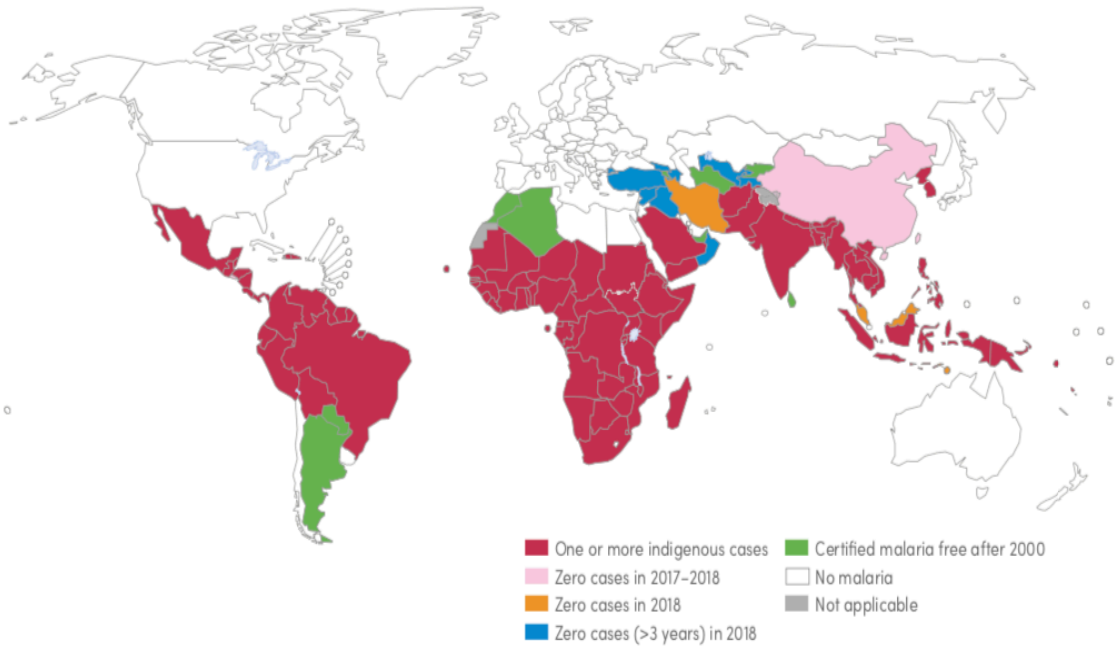

Figure 1 The statuses of countries where malaria was endemic in 2000 are revisited in 2018. Red = countries where malaria is still endemic in 2018; Pink = 0 cases for 2 consecutive years in 2018; Blue = 0 cases for 3 or more consecutive years in 2018; Green = countries certified malaria free after 2000 (WHO, 2019b).

II. Transmission of Malaria: Parasite-Host Interactions

Based on current knowledge, there are over one hundred *Plasmodium* species that can infect a variety of reptiles, birds, and mammals. However, of these hundred or more *Plasmodium* species, five have been confirmed to cause malaria in humans —*P. falciparum, P. malariae, P. vivax, P. ovale,* and *P. knowlesi* (Milner, 2018; Talapko et al., 2019). Other *Plasmodium* species, *P. cynomolgi* and *P. simium*, both of which naturally infects simian primates, have been recently shown to infect humans naturally as well (Ashley et al., 2018; Brasil et al., 2017; Hartmeyer et al., 2019; Ta et al., 2014). Forty percent of the world's population is at risk of contracting malaria from these *Plasmodium* species, and of the species listed above, *P. falciparum* is the deadliest, accounting for the majority of the cases in Africa, the Eastern Mediterranean, and the Western Pacific, traditionally causing the majority of malaria-related deaths (Smith & Jacobs-Lorena, 2010; Tediosi et al., 2017; WHO, 2019b). In 2018, *P. falciparum* caused 99.7% of the estimated cases of malaria worldwide (WHO, 2019b). While *P. falciparum* is the most dangerous, the other species of *Plasmodium* also pose huge threats to the goal of eradicating malaria.

The malaria-causing species, *P. vivax,* is the predominant *Plasmodium* species in the Americas and has the highest burden in Southeast Asia, where it is responsible for half of the cases in this region (WHO, 2019a). *P. vivax* is the cause of 70% or more cases in countries with lower malaria transmission and can also cause severe malaria and malaria-related deaths (Cowman et al., 2016; Shretta et al., 2017). *P. vivax* is known for causing relapsing and chronic symptoms, as the parasite is capable of remaining dormant

in the host's liver for a while before the host exhibits any symptoms. Additionally, gametocytes of *P. vivax* tend to develop before the onset of symptoms, making the disease transmissible before its detection. These characteristics of *P. vivax* make it difficult to eliminate (Dhiman, 2019; Milner, 2018; Shretta et al., 2017). In places where *P. falciparum* and *P. vivax* coincide, *P. falciparum* is more easily managed with malaria control interventions than *P. vivax* (WHO, 2015). In fact, in countries where *P. falciparum* and *P, vivax* coexist, the proportion of *P. vivax* infections tend to increase as the proportion of *P. falciparum* infections tend to decrease, attesting to the difficulty in controlling *P. vivax* malaria (Shretta et al., 2017). Despite the more devastating and deadly pathogenesis of malaria when one is infected with *P. falciparum*, treating other human *Plasmodium* parasites is still just as important in reducing malaria transmission. More severe manifestations of clinical malaria in other *Plasmodium* parasites can be just as deadly, especially to vulnerable populations such as children, pregnant women, and immunocompromised individuals (Milner, 2018; WHO, 2015).

In order to better understand how to optimize malaria control tools, it is important to understand the transmission of malaria to locate targets for interventions and treatments. The *Plasmodium* life cycle and its transmission between mosquitoes and humans have been prominent topics of several malaria reviews, and an overview of the cycle can be seen in Figure 2. *Plasmodium* is a unicellular, obligate parasite, which means that it cannot grow or reproduce without a host (Kaushansky & Kappe, 2015; Kooij & Matuschewski, 2007). This parasite has a complex life cycle that requires both a human host and a female *Anopheles* mosquito to complete its life cycle (Cowman et al.,

2016; Meibalan & Marti, 2017). A human will become host to *Plasmodium* parasites after an infectious mosquito bite, thus starting *Plasmodium* asexual reproduction. Sporozoites, the infectious form of the parasites transmitted from the mosquito to human, will migrate to the liver, where they can undergo a series of mitotic divisions to form merozoites (Cowman et al., 2016; Meibalan & Marti, 2017). Merozoites invade the RBCs in a series of receptor-ligand interactions, which Weiss et al., in their 2015 paper has shown to have specific roles and afflict morphological changes on the RBCs in the process of parasite invasion (Weiss et al., 2015). In *P. vivax* and *P. ovale,* where the parasite typically goes into a long period of dormancy and can relapse over years without parasite detection, the sporozoites form into dormant hypnozoites in the liver until they are reactivated to produce merozoites, a process that is likely activated due to stress (Z. Liu et al., 2011; Milner, 2018; Mueller et al., 2009). In *P. falciparum*, the parasites do not undergo long periods of dormancy and forgo the hypnozoite form (Aly et al., 2009; Cowman et al., 2016). *P. vivax* gametocytes, the mosquito-infecting form of *Plasmodium*, tend to develop before any clinical symptoms appear, making its transmission difficult to prevent and the disease difficult to treat, requiring a 7-14 day anti-malarial treatment course that targets both the liver and blood parasite stages (Ferreira & Castro, 2016; Z. Liu et al., 2011). Given the difficulty controlling transmission on the human side, it is important to emphasize vector control as a suitable method of malaria control.

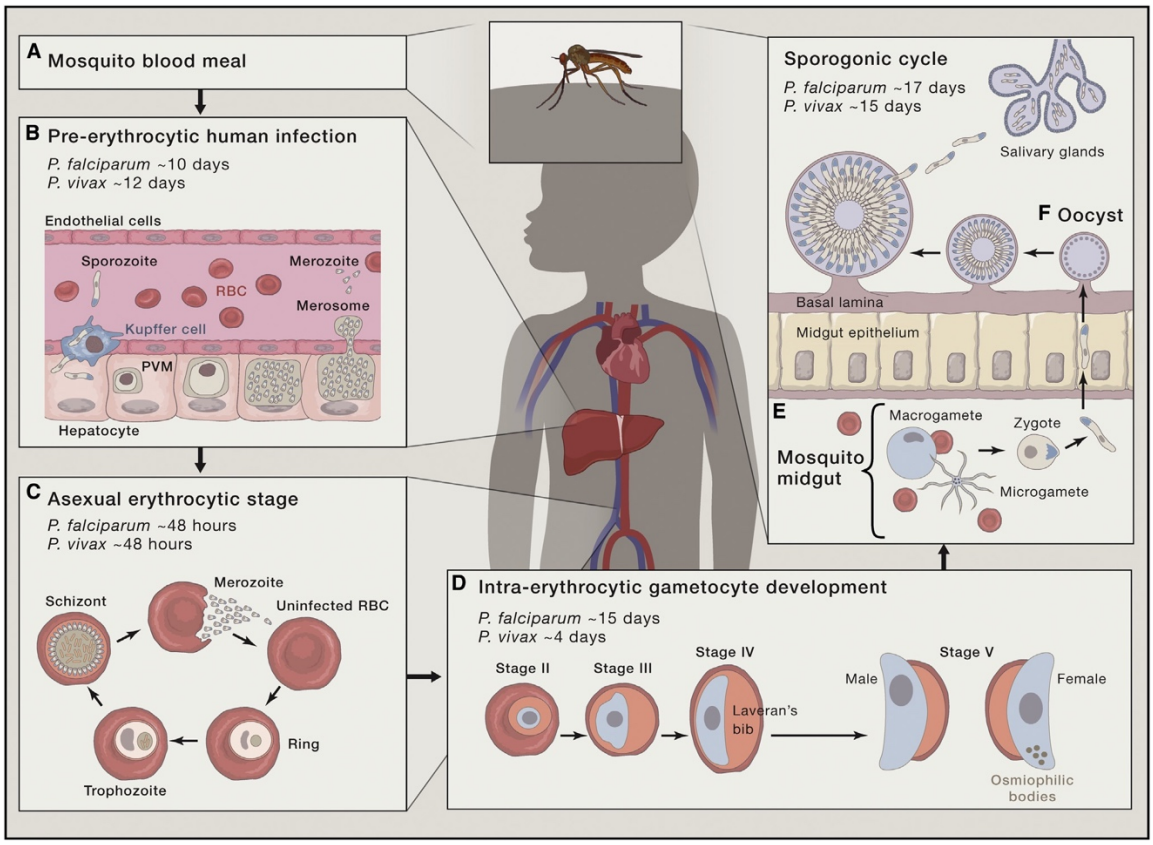

Figure 2 Life cycle of *Plasmodium* parasites in the human host and Anopheline vector. (A) The mosquito takes a blood meal that deposits infectious *Plasmodium* sporozoites in the human. (B) Pre-erythrocytic cycle: Sporozoites infect liver cells in the human, where they can remain dormant as hypnozoites (*P. vivax*) or form merozoites that will infect RBCs. (C) Erythrocytic cycle: Parasites replicate asexually and infect other RBCs, once again repeating the erythrocytic cycle. (D) Gametocytogenesis: Some of parasites will undergo gametocytogenesis, leading to the development of infectious gametocytes that are the infectious components of a mosquito blood meal. (E) Sporogonic cycle: *Plasmodium* gametocytes are activated into macrogametes and microgametes, sexual stages of the *Plasmodium* parasite, and the sexual combination of these two forms will form ookinetes, which develop into oocysts. Oocysts develop in the mosquito midgut and will produce thousands of sporozoites that, when oocysts rupture, will be released into the mosquito salivary gland, allowing mosquitoes to infect humans when it takes a blood meal. Adapted with permission. (Cowman et al., 2016)

Vector control strategies rely on an understanding of mosquito-parasite transmission and interaction. Gametocytes are the infectious form of *Plasmodium* in human blood that is taken up by blood-feeding *Anopheles* mosquitoes to propagate the cycle of malaria transmission. During normal asexual replication, a few parasites will deviate from the normal cycle and form gametocytes, which are sexual forms of the parasite. The infectious bloodmeal is transferred into the mosquito midgut, and within minutes, the gametocytes begin the sporogonic cycle, the sexual replication of *Plasmodium*, by egressing from the human RBCs and differentiating from gametocytes into gametes (Meibalan & Marti, 2017). The formation of gametocytes in humans is typically triggered by environmental stress, which can be due to levels of parasitemia in the host, the presence of host-immune response, and hematological disruptions in the form of anemia, RBC lysis, and increase in erythropoietin and reticulocyte production (Z. Liu et al., 2011). Gametogenesis, the differentiation and the activation of gametocytes into male and female gametes, is triggered by a drop in the temperature in mosquitoes as compared to the temperature in humans and by an increase in xanthurenic acid concentration, along with other factors in the mosquito midgut (Aly et al., 2009; Meibalan & Marti, 2017). The male gametes, also known as microgametes, will exflagellate and produce eight motile microgametes that fertilize female gametes, or macrogametes, to form a diploid zygote (Mitchell & Catteruccia, 2017).

As seen in Figure 3, the zygote will develop into a motile ookinete, allowing the parasite to penetrate the midgut wall into a space between the midgut epithelium and the basal lamina. This process typically occurs between 18 to 24 hours after the blood enters

the midgut. Once the ookinete breaches the midgut epithelium, it will encyst and differentiate into an oocyst, which occurs at around 48 hours after the infectious blood meal (Mitchell & Catteruccia, 2017; Smith & Jacobs-Lorena, 2010). Over the course of the next 2 weeks, the oocysts will undergo sporogony, a process involving rapid cell division and where between 2000 and 8000 sporozoites are produced from within a single oocyst. At around day 14, the oocysts release sporozoites into the hemolymph, from where some sporozoites will eventually invade the salivary glands. Upon the biting of an infectious mosquito, the saliva from the salivary glands, mixed with the sporozoites, will enter the human body, further spreading the cycle of malaria transmission (Mitchell & Catteruccia, 2017; Sinden, 2002; Smith & Jacobs-Lorena, 2010). This process of producing infectious sporozoites occurs over a period of approximately 10 to 14 days after an infectious blood meal, during which only a few females may potentially survive to transmit malaria (Mitchell & Catteruccia, 2017). Sporozoites, after bursting from the oocysts, are released into the mosquito hemocoel and circulate throughout the mosquito body via the hemolymph. The sporozoites, through specific receptor-ligand interactions, will adhere to the salivary glands of the mosquito and invade the salivary glands, making the mosquito capable of transmitting the parasite (Aly et al., 2009). The circumsporozoite protein has an especially important role in sporozoite function, contributing to sporozoite development and mediating the processes of salivary gland and liver cell invasions. As a surface protein on both maturing oocysts and sporozoites, it is a commonly used target of immunofluorescent assays to visualize the development of oocysts and sporozoites (Vlachou et al., 2006). Both ookinete invasion into the midgut and sporozoite invasion

into the salivary glands are known bottlenecks for the parasite survival and should be further studied as potential targets against malaria transmission (Smith et al., 2014; Whitten et al., 2006).

The development of *P. falciparum* in *A. gambiae* mosquito midguts coincides with *A. gambiae* egg development, both of which can only occur with the ingestion of a blood meal (Mitchell & Catteruccia, 2017). In a recent study, Werling *et al*., observed a non-competitive relationship between *A. gambiae* and *P. falciparum*, which was highlighted through the observation of a positive correlation between *Anopheles* egg development and oocyst intensity after an infectious blood meal (Werling et al., 2019). The authors further demonstrated that this relationship was mediated by the mosquito through 20-hydroxyecdysone (20E) steroid hormone signaling. This observation was surprising because previous studies have determined that malaria parasites exert a cost of fitness on *Anopheles* mosquito, thus reducing mosquito survival. A meta-analysis done in 2002 concluded that while malaria parasites do exert a cost of fitness on *Anopheles* mosquitoes, this relationship was mainly seen in unnatural mosquito-parasite pairings (Ferguson & Read, 2002). This study of Werling, *et al.,* however, was carried out with natural mosquito-parasite pairing of *A. gambiae* and *P. falciparum*. The lack of observed fitness costs in this natural mosquito-parasite pairing could explain *P. falciparum*'s impressive spread in Sub-Saharan Africa (Werling et al., 2019). This highlights the importance of 20E and *Anopheles* egg development when looking at *Plasmodium* growth in *Anopheles* mosquitoes.

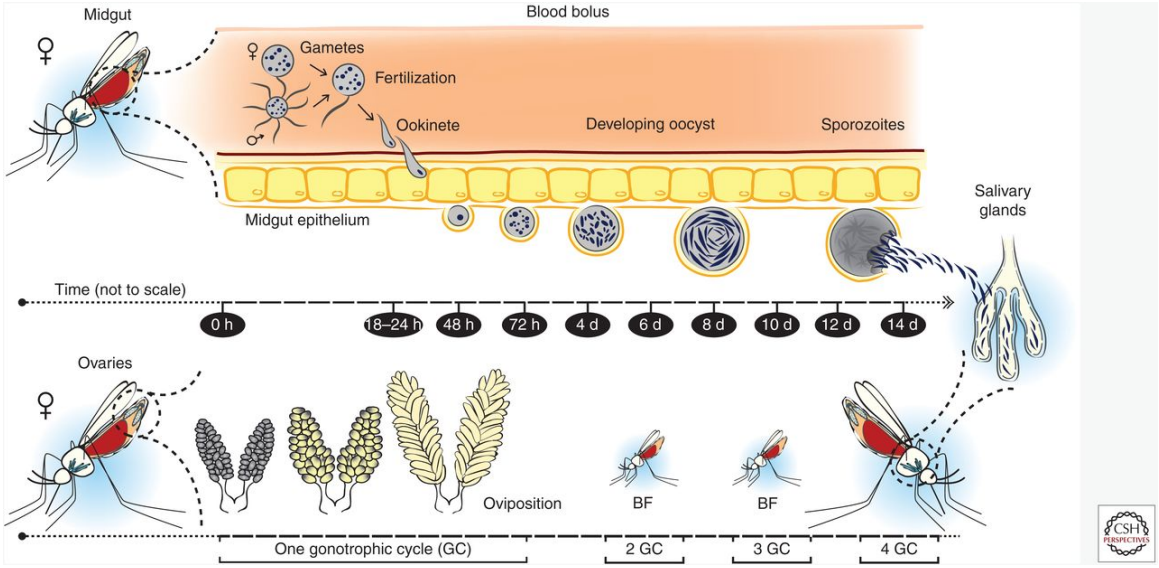

Figure 3 Egg development and *Plasmodium* gametogenesis occur simultaneously. These two processes are both tied to the intake of a blood meal. This figure shows the timeline of mosquito egg development and oviposition, and the timeline of *Plasmodium* development into sporozoites. A female *Anopheline* takes multiple blood meals throughout her life and develops then lays the eggs during each blood meal-induced gonotrophic cycle. At around days 12 through 14, sporozoites have burst from their oocysts, so mosquitoes are capable of infecting human hosts in their next blood meal. Adapted with permission. (Mitchell & Catteruccia, 2017)

The steroid hormone, 20E, mediates the observed positive correlation in egg development and in *P. falciparum* development in *A. gambiae* (Werling et al., 2019). Increased levels of 20E is seen after mating, when the sexual transfer of a mating plug containing male-derived 20E renders the female refractory to mating (Gabrieli et al., 2014). Despite the monandrous mating behavior of *Anopheles* females, mated females are able to develop and lay eggs each time they take a blood meal, which induces the production of 20E in *Anopheles* females (Mitchell & Catteruccia, 2017). When 20E interacts with the receptor complex: ecdysteroid receptor (EcR) and the ultraspiricle, it triggers a number of reproductive signals, such as egg development, lipid accumulation in the ovaries, and egg laying (Swevers, 2019). Given the huge role of 20E on the reproductive characteristics of *Anopheles* mosquitoes, the 20E pathway could be a potential target for controlling vector populations and malaria. However, caution must be taken when targeting the 20E pathway for malaria control, as the results from Werling *et al*., have shown that in silencing EcR, *P. falciparum* tend to have fewer oocysts per midgut but faster oocyst growth and sporozoite development, which could lead to faster malaria transmission; no effect was seen on the prevalence of infection (Werling et al., 2019). Given that normal *Anopheles* females only become infectious in 11-14 days after an infectious blood meal and that a typical female *Anopheles* lifespan is around 2-3 weeks, faster parasite growth can be extremely detrimental to malaria eradication efforts (Clements & Paterson, 1981). With faster parasite development, a greater proportion of female mosquitoes in a given population will be infectious at a given time.

In a recent study, *A. gambiae* females exposed to methoxyfenozide (MET), a 20E agonist, exhibited different effects on *P. falciparum* infection. Virgin female *A. gambiae,* G3 mosquitoes were treated with MET, a compound within the class of dibenzoylhydrazines (DBH). MET successfully decreased the prevalence of mosquitoes infected with NF54 *P. falciparum* when it was topically applied on the thorax of the mosquito. In addition to its activity against malaria infections, increasing concentrations of MET led to decreased egg development, apoptosis of ovarian follicles, reduced lifespan, and prevention of virgin female *Anopheles* mosquitoes from initiating mate-seeking activities (Childs et al., 2016). In a 2012 study, DBH compounds including MET, were shown to have insecticidal activity against *Anopheles* larvae. MET was shown to have the greatest toxicity when compared to the other compounds tested but is non-toxic to humans (Morou et al., 2013). MET's larvicidal qualities along with its observed effects against *P. falciparum* infection in *A. gambiae* females, made it a candidate in the search for new compounds to use in malaria and vector control.

III. Malaria Control and Resistance to Insecticides and Antimalarials

Malaria is one of many vector-borne diseases, with some other examples being chikungunya, West Nile virus, and Zika virus. In a 2019 review published by *Nature Microbiology,* Shaw and Catteruccia described vector control as the most effective method of stopping mosquito-borne diseases, which is in concordance with the WHO's belief that mosquito control can prevent and bring malaria transmission close to zero (Shaw & Catteruccia, 2019; WHO, 2020a). In malaria-endemic regions, insecticide-

treated nets (ITNs), indoor residual sprays (IRS), and artemisinin combination therapies (ACTs) have all contributed to the decline in transmission (WHO, 2019b). Their contributions are explored in a 2015 *Nature* paper, where Bhatt *et al*., modeled the effects of using these three interventions on the decrease in the prevalence of malaria cases between 2000 and 2015. All three interventions contributed to the decrease in the prevalence of disease when compared to a counterfactual (i.e., no intervention) trend, and ITNs had the greatest predicted impact in contributing to the number of cases averted and in decreasing the prevalence of the disease. In fact, ITNs are predicted to have been responsible for as much as 68% of the decrease in malaria cases between 2000 and 2015 (Bhatt et al., 2015). ITNs and IRS are the main and most effective forms of vector control currently used, and ACT is currently the most effective treatment against *P. falciparum* in humans. Although these tools have greatly shaped malaria control strategies, more research still needs to be done to fully optimize their effects.

3.1 Insecticide-Treated Nets and Long-Lasting Insecticide-Treated Nets

Vector capacity estimates the ability of a vector to transmit pathogens to humans. Six factors are involved in this equation—vector density, vector biting rate, vector competence, vector daily survival probability, pathogen incubation period, and vector lifetime survival. ITNs and IRS work to decrease the factors mentioned in the vector capacity equation to limit the mosquito's capacity to transmit malaria (Shaw & Catteruccia, 2019). ITNs provide a protective barrier for people sleeping under them, but they have also been shown to have a community effect, where members of the

community that aren't sleeping under a net are also protected due to decreased mosquito survival (WHO, 2019b). While one study observed the community effect in non-net users, they heavily emphasized the need for better surveillance to prove that this effect does exist; another study did not observe a community effect in non-net users (Komazawa et al., 2012; Quiñones et al., 1998). Nonetheless, when ITNs are used, the prevalence of *P. falciparum* infections was reduced by 17% compared to when no nets were used, and when compared to non-treated nets, ITNs reduced the prevalence by 10% (Pryce et al., 2018). This shows that ITNs have improved the health outcomes of at-risk populations in malaria-endemic regions.

ITNs are traditionally impregnated with a class of insecticides called pyrethroids, which are commonly used because they are shown to have fewer toxic effects at certain doses against humans than other insecticide classes (Zaim et al., 2000). A recent study, however, suggested that these pyrethroids might not be free of adverse effects at low doses and should be used with caution (Chrustek et al., 2018). One of the newer forms of ITNs are long lasting insecticide-treated nets (LLINs). LLINs are impregnated with the insecticide and must survive 20 washes and 3 years of use under field conditions; this makes re-treating ITNs unnecessary (de Oliveira Sousa et al., 2019). Two common insecticide formulations are used on LLINs: 1) pyrethroid insecticide only, such as the OLYSET® net consisting of only permethrin, and 2) pyrethroid insecticide with piperonyl butoxide (PBO) synergist, such as the OLYSET Plus® net consisting of permethrin and PBO (WHO, 2020b). The OLYSET® nets are only two nets amongst many other nets with different combinations of insecticide and PBO. PBO has been used

for the past few decades in agricultural settings as a synergist to enhance the activity of insecticides through two mechanisms: 1) inhibiting metabolic enzymes in mosquitoes, such as P450s and non-specific esterases, and 2) enhancing the insecticide penetration of mosquito cuticles (Bingham et al., 2011). A study showed that the addition of PBO to the LLINs completely restored susceptibility of a sample of *A. funestus* despite heavy pyrethroid resistance in these mosquitoes (Churcher et al., 2016; Sinka et al., 2012). Despite its success in enhancing insecticide uptake, PBO is also a possible human carcinogen, thus, making it potentially dangerous as a compound in LLINs and must be used cautiously. Taking into account PBO's potential carcinogenic effects, a study done by Marchand, *et al.,* showed that vegetable oils, such as rapeseed methyl ester (RME), can be just as effective as PBO in working as a synergist for insecticide uptake (Marchand et al., 2018). In a recent study from Lees, *et al.*, researchers showed that RME enhanced the tarsal uptake of several insecticides, activating 11 compounds—previously ineffective in tarsal contact assays —with the addition of RME (Lees et al., 2019). RME has potential to enhance tarsal uptake and could potentially be a solution to making previously ineffective insecticidal compounds, effective.

Previously, pyrethroids were the only insecticide class used on bed nets and the most popular insecticide for IRS. Recently, other compounds have been explored: chlorfenapyr, a pro-insecticide that becomes active in the host, and pyriproxyfen, a juvenile growth hormone mimic that is commonly used as a larvicide, have been shown to be effective insecticides against *Anopheles* mosquitoes (N'Guessan et al., 2016; Ngufor et al., 2014). In N'Guessan's study, they formulated an LLIN mixture,

Interceptor® G2, that combines a pyrrole, chlorfenapyr, and a pyrethroid, alpha-cypermethrin, and they tested this mixture against pyrethroid-resistant *A. gambiae*. The results were promising, showing that the mixture killed 71% of permethrin-resistant *A. gambiae* as compared to an alpha-cypermethrin-only mixture that killed only 20% of permethrin-resistant *A. gambiae*, and after 20 washes, the mixture killed 65% of permethrin-resistant *A. gambiae* (N'Guessan et al., 2016). As seen from the incomplete killing by the Interceptor® G2 on pyrethroid-resistant *A. gambiae*, it may be that there is already some form of resistance against the chlorfenapyr, despite its higher efficacy in decreasing survival. This further emphasizes the need for more research on insecticides and insecticide resistance to improve the efficacy of control tools. The use of pyriproxyfen in a new LLIN product, the Olyset Duo®, consisted of a combination of pyriproxyfen and permethrin, a pyrethroid compound (Ngufor et al., 2014). They observed that Olyset Duo® LLINs provided greater protection for children participating in their study in Burkina Faso relative to pyrethroid only LLINs, reducing a normal 85 average bites per infection season to only 42 infective bites per infection season (Tiono et al., 2018). Both of the new LLINs take into account the recommendation to not rely solely on pyrethroids and to combine different classes of insecticides in LLIN formulations (Weedall et al., 2019). Ultimately, the same principles apply for IRS: There needs to be more diverse combinations of insecticide classes in IRS mixtures. LLINs and IRS must use insecticides that both work on mosquitoes and have limited adverse effects on humans, hence their strong reliance on pyrethroids. Yet, there are 5 other classes that have been recommended for use against adult mosquitoes: organophosphates,

carbamates, organochlorines, and pyrroles, and phenyl pyrazoles, that must still be better explored (Liu, 2015). However, there have been increasing reports of resistance against these classes of insecticides as well (Ranson & Lissenden, 2016). Other forms of compounds, such as insect growth regulators and known agricultural pesticides, are also currently being explored for malaria control uses. The heavy reliance on pyrethroids in the past has built heavy resistance against the insecticide in many places worldwide, rendering the LLINs and IRS less effective, so it is important that more research must be done to find alternatives compounds for vector control tools.

Despite the number of cases averted by these interventions, the incidence of malaria cases has remained stagnant since 2014, which could be due to increasing resistance against antimalarials and insecticides (WHO, 2019b). There are many ways insecticide resistance can occur in mosquitoes, three of which are common: 1) metabolic resistance, 2) cuticular resistance, and 3) target site resistance; a fourth mechanism of resistance, behavioral resistance, has been observed as well (Ranson et al., 2011). Metabolic resistance occurs when enzymes that detoxify the insecticides are overexpressed in the presence of stressors. Commonly seen detoxification enzymes include P450 monooxygenases, glutathione-S-transferases, and carboxylesterases (Toé et al., 2015). The cytochrome P450 genes are often overexpressed in pyrethroid resistant mosquitoes. Mosquitoes with the cytochrome P450 pyrethroid-resistant allele *CYP6P9a_R*, which is found mainly in a major African malaria vector *A. funestus*, showed increased survival and success in blood feeding despite the presence of pyrethroid insecticides found on ITNs. This allele has evolved to build metabolic

resistance against insecticides (Weedall et al., 2019). Cytochrome P450s are also associated with cuticular resistance, which occurs when mosquitoes are not able to take up insecticides due to the insecticides' inability to pass through the mosquito cuticular barrier. It is involved in the production of cuticular hydrocarbons, the compounds that make up the mosquito cuticles. Increased cuticular hydrocarbons provide a thicker barrier against insecticides, making insecticides unable to enter the mosquito through tarsal contact (Balabanidou et al., 2016).

Tarsal contact is a key component for the effectiveness of IRS and LLINs, and cuticular resistance, which directly affects the ability to take up insecticides via tarsal contact, is a threat to the effectiveness of these tools. Target site resistance, which is commonly called knock-down resistance, occurs when the target sites become insensitive to the insecticides due to changes in the target site structure or due to point mutations (Liu, 2015). Resistance is extremely detrimental to the progress made in malaria eradication in the past decades, and more research must be done to understand the implications of insecticide resistance and find better tools to overcome this barrier.

These forms of insecticide resistance have built up over time through the poor management of insecticide-use and using compounds that focus on the same target, rather than a diversity of targets. Vector control through insecticides must use a variety of compounds that have assorted targets to ensure mosquito death and resist building resistance. In light of the resistance that has built up against insecticides in LLINs and IRS, researchers are now looking towards repurposing already-existing insecticides that

were not previously used for vector control (Lees et al., 2019). There is still much work to do be done on finding different classes of compounds to use on vector control tools.

3.2 Artemisinin Combination Therapy and Artemisinin Resistance

Resistance has not only developed in the mosquitoes but also in the parasites. A good example of this is artemisinin resistance. ACT is currently the most effective drug therapy used to treat *Plasmodium* infections. It uses the drug artemisinin in combination with partner drugs, such as lumefantrine, mefloquine, amodiaquine, or sulfadoxine/pyrimethamine, and has been successful in treating uncomplicated malaria, leading to a 18% decrease in the incidence of malaria cases between 2010 and 2016 (Ouji et al., 2018). However, the poor administration of artemisinin treatments, often using monotherapies of artemisinin-only drugs rather than ACT, has led to the spread of artemisinin resistance throughout the Greater Mekong Subregion (Miotto et al., 2013). In 2009, the first confirmed case of artemisinin resistance was seen in Western Cambodia. (Fairhurst & Dondorp, 2016). In a 2014 study, the median parasite clearance half-life after taking an ACT in the Democratic Republic of Congo was 1.9 hours and at the Thailand-Cambodian border was 7 hours, which shows the effect of resistance to ACT (Ashley et al., 2014). There have been no signs of artemisinin resistance outside of the Southeast Asia region (Rosenthal, 2018). However, the spread of artemisinin resistance is always a concern.

Artemisinin resistance in *P. falciparum* is caused by a mutation in the kelch-13 gene, of which the C580Y mutation is the most common mutation (Kobasa et al., 2018).

A 2017 study described kelch-13 and pfs-47, a *P. falciparum* surface membrane protein, to be genetically linked (Molina-Cruz et al., 2017). Pfs-47 helps *P. falciparum* evade the *Anopheline* immune system. (Molina-Cruz et al., 2017; Molina-Cruz & Barillas-Mury, 2014). Thus, *P. falciparum* with kelch-13 mutations that lead to artemisinin resistance are likely to continue evading the *Anopheline* immune system and persist in the population. While ACT is no longer be as effective as it once was, vector control methods can be supplementary in controlling the transmission of artemisinin resistant parasites.

3.3 Atovaquone and Similar Compounds

A recent study published in *Nature* shows that antimalarials can be taken up through tarsal contact to eliminate *P. falciparum* infections in *A. gambiae*. In this paper, Paton et al., exposed *A. gambiae* females to ATQ before feeding them an infectious *P. falciparum* blood meal. A dilution series was carried out to observe the effects in prevalence of infection and in oocyst intensity of *P. falciparum* after a 6-minute exposure to ATQ through tarsal contact (Figure 4). The study found that ATQ stopped a majority of the parasites from developing past the zygote stage, so at 100µmol/m^2, no infection was seen in the midgut. Paton, *et al.*, also saw a decrease (Figure 5) in the prevalence of infection when using the same exposure model with hydramethylnon (HYD) and acequinocyl (ACE) (Paton et al., 2019). ATQ is an apicomplexan-specific inhibitor of cytochrome bc_1, which is found in the inner membrane of the mitochondria (Srivastava et al., 1997). ATQ's function as a cytochrome bc_1 inhibitor not only blocks ATP synthesis by inhibiting an integral part of the electron transport chain but also inhibits *de novo*

pyrimidine synthesis, an important and necessary process in *P. falciparum* DNA replication. It was shown that in human stages, *P. falciparum* development relies cytochrome bc_1 for *de novo* pyrimidine synthesis and not for ATP synthesis (Painter et al., 2007). Thus, ATQ's function in inhibiting the development of *P. falciparum* zygotes can be due to a lack of ATP, the inability to replicate DNA, or both. Another study showed that the inhibition of type II NADH:ubiquinone dehydrogenase, also a part of the electron transport chain, prevented ookinete maturation into oocysts (Boysen & Matuschewski, 2011). This further supports that the electron transport chain, an essential part of *P. falciparum* development and growth in the mosquito stages, would make a good target for malaria control.

Like many of the compounds mentioned before, resistance has already developed against ATQ in *Plasmodium* parasites. ATQ resistance has previously been observed in human stage *Plasmodium* parasites; however, a 2016 study showed that mutations conferring resistance to ATQ in *P. berghei*, a rodent malaria parasite caused complete arrest of developing parasites during the oocyst stage, suggesting ATQ resistance mutations are non-transmissible (Goodman et al., 2016). Moreover, cytochrome bc_1 mutations, associated with ATQ resistance, have been shown to be linked to decreased fitness in *P. falciparum* because of the mutation's effect on the parasite's respiration (Goodman et al., 2016; Peters et al., 2002). From these studies, ATQ resistance exerts costs on *Plasmodium* fitness, and this makes ATQ and other cytochrome bc_1 inhibitors good candidates for tool development. HYD and ACE were used in Paton's study because of their similar target as ATQ: cytochrome bc_1, and they have been used as

pesticides in the past (Hooper-Bùi & Rust, 2001; Paton et al., 2019). While the 2019 study has shown that ATQ is effective in eliminating parasite infection in mosquitoes when the mosquitoes are exposed before an infectious blood meal, it is still unclear what the limits of atovaquone-treatment of *Anopheline* mosquitoes exposed to *P. falciparum* are. Further research must also be done to understand ATQ's limit as a malaria vector control tool and on using ATQ with other vector control tools that can bypass cuticular resistance associated with tarsal contact.

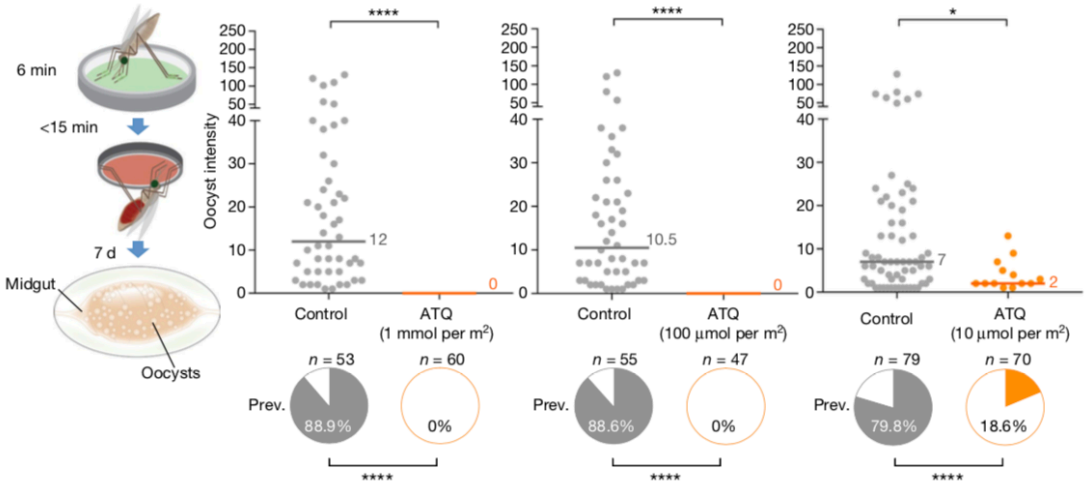

Figure 4 Female *A. gambiae* mosquitoes are exposed to ATQ for 6 minutes in a dilution series assay (1 mmol ATQ, 100 µmol ATQ, and 10 µmol ATQ) and given an infectious blood meal right after the ATQ exposure. Midguts are dissected and imaged for the prevalence and intensity of oocysts at day 7 pIBM. Adapted with permission. (Paton et al., 2019)

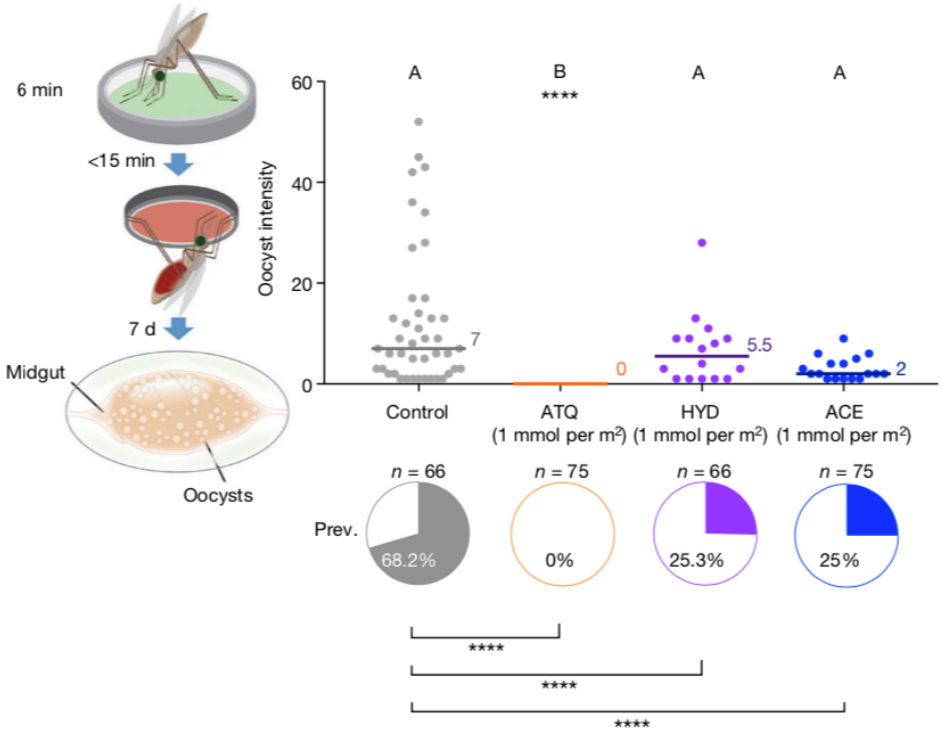

Figure 5 Female *A. gambiae* mosquitoes were exposed to 1mmol per m^2 of either ATQ, HYD or ACE, for 6 minutes and then fed an infectious blood meal. The midguts are dissected, and the oocyst intensity and prevalence of infection are measured on day 7 pIBM. Adapted with permission. (Paton et al., 2019)

3.4 Attractive Toxic Sugar Baits

Attractive toxic sugar baits (ATSBs) are an increasingly popular tool for controlling mosquito populations. Especially since ITNs and IRS both target mosquitoes that feed and rest indoors, it is limited in its uses because not all malaria-carrying mosquitoes feed and dwell indoors (Ranson et al., 2011). Additionally, there has been anecdotal reports of behavioral resistance, claiming an increase in outdoor-feeding mosquitoes due to the increase in insecticide-use indoors, this was later supported by an individual-based modelling study in 2017 (Bøgh et al., 1998; Ranson et al., 2011; Zhu et

al., 2017). Sugar baiting can target both outdoor and indoor environments and behavior of the mosquito to deter certain behaviors (such as biting) and can induce mosquito death. Insect baiting is not a new concept, having existed since before 77 AD, and adding attractants and feeding stimulants are also concepts seen in the past (Fiorenzano et al., 2017). However, with the goal of distributing ATSBs as a worldwide effort to reduce mosquito-borne diseases, ATSB-use needs to be further studied to understand how to fully optimize the tool and reduce its effects on non-target insects.

ATSBs can be a powerful tool for malarial control because, unlike other tools currently in circulation, ATSBs target both male and female mosquitoes rather than just the biting adult females, showing significant reduction in both population's survival after ATSB exposure (Maia et al., 2018; Qualls et al., 2015). In many studies, ATSBs have successfully reduced the mosquito population and decreased the number of older female mosquitoes, a population known to have a greater chance of malaria infection, and the number of sporozoite-transmitting mosquitoes (Qualls et al., 2015; Traore et al., 2020). Mosquitoes acquire their sugar meals from a variety of plants, mainly flowers, fruits, and seed pods. In a study done on the attractive component of ATSBs, it was observed that ripe guava and honey melons were especially attractive to *A. gambiae* (Müller et al., 2010). Many studies have observed species-specific preferences in the field, with growing evidence supporting the relationship between plant sugar availability and mosquito fitness (Airs et al., 2019; Foster, 1995). While many studies have been done to assess sugar preference of the mosquitoes, little research has been done on how sugar composition is distributed in the mosquito gut system and how a sugar composition

affects the attractiveness of ATSBs in *A. gambiae*. In a 2019 study published in *Acta Tropica*, Airs, et al. showed that most sugars distributed into the ventral diverticulum, also known as the crop, in *Aedes aegypti* mosquitoes, and in those same mosquitoes, arabinose, lactose, and cellobiose significantly decreased their survival when compared to mosquitoes fed with sucrose (Airs et al., 2019). When using ATSBs to target *A. gambiae* populations and *P. falciparum* infections, knowing the sugar composition and how different sugars distribute could elucidate where the drugs and insecticides used in ATSBs will end up and whether there are any unexpected reactions between the attractive sugar source and the insecticides/drugs.

In many villages where malaria is endemic, LLINs and IRS are not sufficient to controlling malaria, and a 2017 study concludes that ATSBs should be incorporated in vector control given that there was significant reduction when using both LLINs and ATSBs as compared when using only LLINs (Zhu et al., 2017). One of the dangers of using ATSBs is its non-specific targeting of other insects that might be beneficial to the environment, but a 2017 review concluded that the effects of ATSBs on non-target arthropods are limited (Fiorenzano et al., 2017). Nonetheless, it is important to exercise caution in deciding what kinds of insecticides and drugs to include in ATSBs. More studies must be done on diversifying the insecticides used in ATSBs, just as current trends are increasing insecticide diversity in LLINs and IRS.

SPECIFIC AIMS

As vector control is an effective method of malaria transmission control and prevention, it is important to place importance on testing compounds that could be used in vector control tools. Two tools that are of great interest are ITNs, which requires tarsal contact for the exposure compound to be effective, and ATSBs, which requires ingestion of a compound-treated sugar meal to be effective. This thesis will be looking at the natural parasite-mosquito pairing, *P, falciparum* and *A. gambiae*.

Aim 1: Determine the effects of ATQ-treated sugar feeding on *P. falciparum* infection in *A. gambiae* when mosquitoes are treated before and after an infectious blood meal.

Aim 2: Determine the effects of HYD-treated sugar feeding on female *A. gambiae* survival and on *P. falciparum* infection in *A. gambiae* when mosquitoes are treated before an infectious blood meal

Aim 3: Determine the effects of MET exposure through tarsal contact on *P. falciparum* infection in *A. gambiae* and on *A. gambiae* egg development when mosquitoes are exposed before an infectious blood meal and the effects of MET-treated sugar feeding on *A. gambiae* egg development when mosquitoes are exposed before a blood meal.

These 3 aims will help define the limits of the three compounds: ATQ, HYD, and MET, on their efficacy and potential use in vector control tools through either sugar feeding or tarsal contact tools.

METHODS AND MATERIALS

I. Insect Lines and Rearing

The mosquitoes used in these experiments were reared in the insectary at the Harvard T.H. Chan School of Public Health. Wild type *A. gambiae,* G3, mosquitoes were used. All *A. gambiae* mosquitoes were kept in an insectary at 27°C and 80% relative humidity on a 12-hours light and 12-hours dark cycle to replicate field-like conditions. Adults were kept in large colony cages (Bugdorm Megaview Science, Taichung, Taiwan) and provided 10% w/v glucose solution *ad libitum*. All cages were provided with water and a plastic cover to maintain stable humidity. For colony maintenance, 5-7 days after eclosure from their pupae form, adult female mosquitoes were provided with human blood meals using an artificial membrane feeding system (Hemotek, Accrington, UK). All human blood samples were donated voluntarily (Research Blood Components, Boston, MA).

II. *P. falciparum* infection assays

For infection, female mosquitoes were separated from the males as pupae in order to obtain virgin adult female mosquitoes. Non-virgin adult female mosquitoes can be taken from a mixed cage using a HEPA-filter mouth aspirator (J.W. Hock, Gainesville, FL). *P. falciparum* NF54 parasites were used in all infections (BEI resources, Manassas, VA). The parasites were cultured in asexual stages between 0.2% and 2% parasitemia at 37°C in human RBCs at 5% hematocrit supplied by the Interstate Blood Bank (Memphis, TN). This parasite culture solution was comprised of RPMI 1640 with 25mM HEPES, 10

mg/L hypoxanthine, 0.2% sodium bicarbonate, and 10% heat-inactivated human serum (Interstate Blood Bank, Memphis, TN) under a gas mixture of 5% O_2, 5% CO_2, balanced N_2 for up to 8 weeks (Ifediba & Vanderberg, 1981; Trager & Jensen, 1976).

Gametocytogenesis was induced before infecting the mosquitoes. This was done by raising parasitemia above 4% and incubating the cultures for 14-20 days to accrue stage IV and V male and female gametocytes. The media was replaced daily. Mosquitoes were placed in small cages (Bugdorm, Megaview Science, Taichung, Taiwan) and fed infectious blood in a sealed, secured infection glovebox through a glass, water-heated membrane feeder for 60 minutes (Hemotek, Accrington, UK). The female mosquitoes that were visibly unfed were vacuum aspirated into 80% ethanol and discarded within 12 hours of the infectious blood meal. The remaining mosquitoes were left in the secure glovebox and provided 10% w/v glucose solution *ad libitum,* refreshed every 3-5 days, unless otherwise specified.

In most experiments, the mosquitoes were aspirated on day 7 after an infectious blood meal, which is sufficient for looking at oocyst development and prevalence of infection. In specific experiments however up to 10 days after infection may have been examined. When collecting salivary glands for sporozoite data, mosquitoes were aspirated into RPMI medium on day 14 or later after an infectious blood meal.

For observing the number and size of oocysts in female mosquito midguts, the mosquitoes in 80% ethanol were incubated for 10 minutes at -20°C and then removed from the ethanol and put into 1x PBS (made from a 10x PBS and ddH_2O solution). The midguts were dissected from the mosquito using a pair of forceps and a surgical needle

and stained with 2mM mercurochrome in ddH_2O for 15 minutes. After staining, the guts were mounted on glass microscope slides in 0.2mM mercurochrome and examined at an object of 40x on an inverted compound light microscope (Olympus).

For observing the number of sporozoites, infected mosquitoes were decapitated in the secure glovebox before removal from the box. The salivary glands were dissected in RPMI medium from the mosquitoes and placed into Eppendorf tubes with RPMI medium. The salivary glands were individually ground by pestles and subsequently spun via centrifugation at 8000 g for 10 minutes at 4°C to release the sporozoites. The supernatant fluid was disposed of and the remaining pellet was resuspended in a known volume of RPMI or 1x PBS. A 10µL solution of the sporozoites suspended in fluid was pipetted into disposable hemocytometers (Fischer Scientific), and sporozoites in 0.1µL were counted at 400x magnification using phase-contrast on an inverted compound microscope (Olympus). The total sporozoites per mosquito was calculated from the 0.1µL sample.

III. Exposures (Tarsal contact and sugar feeding)

The compound MET (Sigma-Aldrich), used in tarsal contact exposures was dissolved in acetone, a volatile vehicle, at stock concentrations of 3-10 mg ml^{-1} (0.3-1% w/v). These stock solutions were diluted to create the desired concentrations needed for the experiments. A known volume of the solutions containing the MET was added to 500µL excess of acetone and applied onto a glass petri dish (area = 0.0283 m^2). In the case where RME was added, the RME, also dissolved in acetone to a 100µmol/L

concentration, was added to excess of acetone before a known volume of MET dissolved in acetone was added. The treated plates were left for at least 4 hours on a lateral shaker until the acetone evaporated. Control plates were also given acetone with no compounds added, and the same treatment was applied. A plastic cup was inverted over the glass dish, and a flap was cut into the base of the cup in order to aspirate mosquitoes into contraption. A HEPA-filter mouth aspirator (J.W. Hock, Gainesville, FL) was used to aspirate 25-30 mosquitoes onto the glass dish, and the mosquitoes were incubated for 60 minutes. The plates were agitated every 15 minutes to make sure the mosquitoes were landing on the plate rather than clinging onto the untreated plastic cup. Once the exposure was completed, the mosquitoes were transferred into a small cage by opening the entire dish contraption inside of the cage.

Compounds, ATQ, MET, and HYD used in sugar feeding assays were dissolved in DMSO to form a stock concentration of 20nM, and dilutions were made from the stock solution before being added to 10mL of 10% w/v glucose solution. The final glucose solutions had 0.5% DMSO, which was taken from the stock solution within which the compounds were dissolved. Controls were given a similar 10 mL of 10% w/v glucose solution with 0.5% DMSO. Cotton pads were soaked with the 10 mL sugar solutions and placed in small plastic dishes. When working with non-infected mosquitoes, mesh netting was placed on top of these cotton pad dishes, and the dishes were placed into the mosquito cages. When working with infected mosquitoes, the sugar dishes were inverted over the top of the cage without any mesh netting. The sugars were left on or in the cage

from anywhere between 24 hours to 14 or more days, depending on the experiment, and they were changed every 3-4 days.

IV. Survival assays

Survival was assessed for HYD sugar feeding exposures. Around 40 female mosquitoes were placed in small cages and exposed for 24 hours to a dilution of HYD (100μM, 10μM, and 1μM) and subsequently provided 10% w/v glucose solution. Water was also given after the exposure was removed for added humidity. Survival was measured daily 24 hours after the exposure was administered by counting and removing all of the dead mosquitoes in the cage at a 24-hour time point. The experiments continued until all of the mosquitoes died.

V. Egg development and Oviposition assays

Female virgin mosquitoes were exposed to MET for an hour immediately before their infectious blood meal via tarsal contact. A blood meal, infectious or noninfectious blood, was given to the mosquito using the methods above, and non-blood fed female virgin mosquitoes were removed. After 7 days, the gravid female virgin mosquitoes were collected, and their ovaries were dissected out in 1x PBS. Gentle agitation allowed the ovaries to release the developed eggs, and the eggs were counted. Virgin females must be used in order to prevent egg laying before counting the eggs at day 7. For oviposition experiments, female virgin mosquitoes were exposed to 1mmol/m^2 MET via tarsal contact and given a blood meal, using an artificial membrane feeding system, 24 hours

after the exposure. After 48 hours, the mosquitoes were placed in oviposition cups, a paper cup with filter paper lining the side and a bit of water at the bottom of the cup. The mosquitoes were provided 10% w/v glucose solution, and five days after the blood feed, the laid eggs and eggs still inside the mosquito ovaries were counted.

VI. Measurement of Ecdysteroid levels

Groups of female virgin mosquitoes were exposed to 1mmol per m^2 MET for an hour, 24 hours before their blood feed or right before receiving their blood meal using an artificial membrane feeding system. At 24 hours post blood meal, when 20E levels are typically the highest, the female virgin mosquitoes were decapitated, and the rest of the bodies were stored in 80µL of 80% methanol at -80°C. The 20E levels were measured using the 20E enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI) using the protocol provided by the kit. The EIA plates were read on a spectrophotometer at 412 nm at 90 minutes.

VII. Statistical Analysis

The statistical analysis performed in the GraphPad Prism v. 8.1.2. for MacOSX, except for the survival analysis, which was done using JMP Pro 14 (SAS Institute). When comparing the median oocyst intensity (or intensity of infection) between two groups, a Mann-Whitney mean ranks test was used due to its non-parametric nature. When performing multiple comparisons on the median oocyst intensity amongst various groups, a Wilcoxon with Dunn's post-hoc multiple comparisons test was used. An unpaired T-

test was used to compare egg development counts per mosquito and sporozoites counts per mosquito between two groups due to their parametric nature. The ordinary one-way ANOVA along with the Sidak's multiple comparison test was used for multiple comparisons for parametric data. For comparing prevalence of infection between two groups, a chi-squared test was used to determine the significance of the infection. For comparing the prevalence of infection amongst multiple groups, a Fisher's exact test that was corrected for multiple comparisons (Bonferroni) was used. Chi-squared analysis was used to analyze the differences in survival amongst the various groups of HYD-exposed mosquitoes and the controls. A log-ranked Mantell-Cox test was used to analyze the differences in median time-to-death amongst the treatment and control groups.

RESULTS

I. ATQ exposure through sugar feeding on *A. gambiae* before an infectious blood meal resulted in a dose-dependent decrease in *P. falciparum* prevalence of infection.

Previous research has shown that ATQ has an antimalarial effect on infected mosquitoes through tarsal contact (see Figure 4, above). While these effects can be used to improve current malarial control tools, it is still unclear to what extent ATQ can be used in malaria control tools other than LLINs. For this reason, ATQ's effects on parasite development in *A. gambiae* females was studied in ATSBs. As females take essential sugar meals prior a blood meal, the effects of ATQ-treated sugar feeding prior to an infectious blood meal was studied. Mosquitoes, starved for 24 hours to incite hunger, were exposed to ATQ-treated sugar for 24 hours *ad libitum* (Figure 6). ATQ was dissolved in 0.5% DMSO to solubilize the compound, making it easier to dissolve in the 10% w/v glucose solution. Shortly after the 24-hour exposure, the females were fed a NF54, *P. falciparum* blood meal. Non-blood fed females were removed after the feed to remove chances of a negative infection phenotype (no oocysts in the midgut) due to nonfeeding. At day 7 pIBM, the oocysts were visible when stained with mercurochrome, and the oocysts were counted.

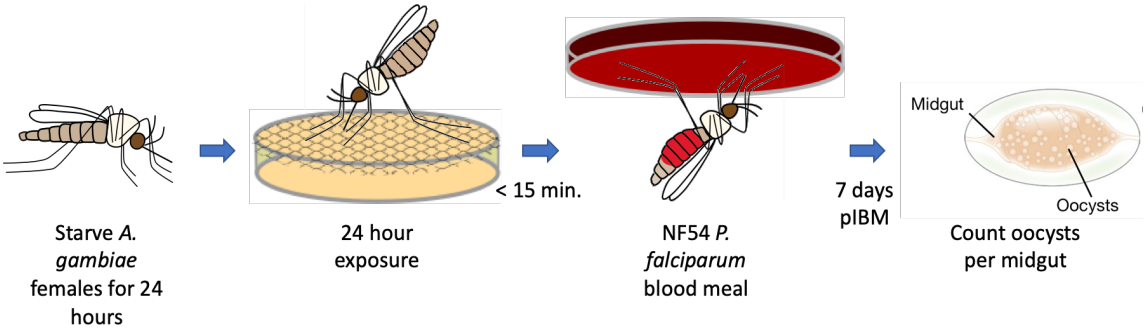

Figure 6 Pre-infection sugar-feeding assay: *A. gambiae* females are starved for 24 hours before they are fed a compound-treated sugar meal for 24 hours. Immediately after removing the exposure, *A. gambiae* females are given an infectious blood meal, and 7 days pIBM, the prevalence of *P. falciparum* infection and *P. falciparum* infection (oocyst) intensity is measured.

Previous unpublished data (Figure 7) from our lab has shown that feeding female *A. gambiae* 100µM ATQ + 0.5% DMSO in a 10% glucose solution *ad libitum* for 24 hours before an infectious blood meal leads to a complete block of *P. falciparum* infection by day 7 (two-sided Fisher's Exact Test, $n = 111$, $p < 0.0001$). This result is similar to the inhibition of infection seen in tarsal contact when *A. gambiae* females were exposed before an infection.

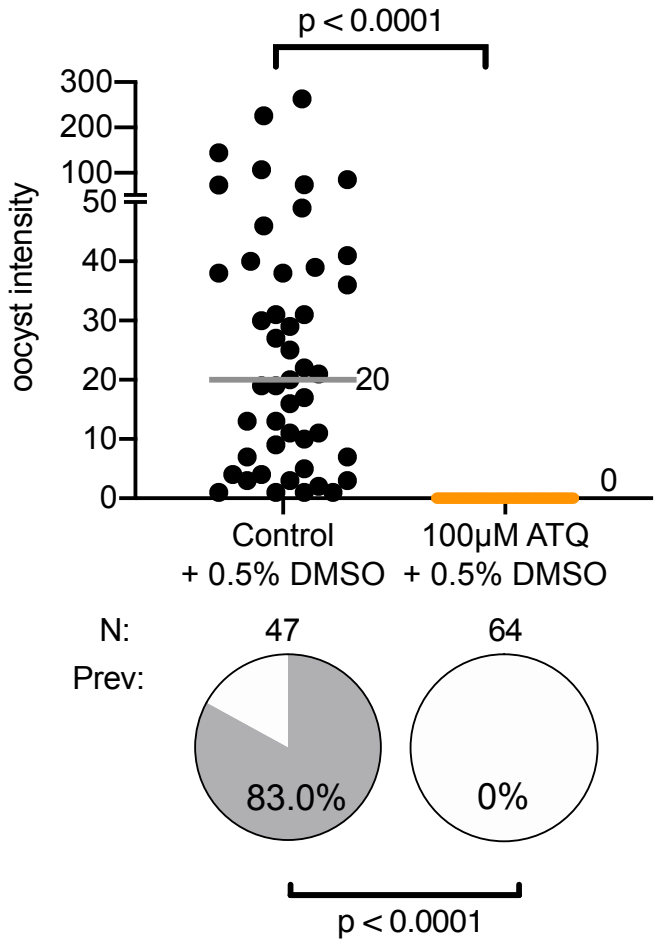

Figure 7 Preliminary data (from Douglas Paton, personal communication, 2019) that shows the oocyst intensity and prevalence of infection of *P. falciparum* after *A. gambiae* females are exposed to 100μM ATQ + 0.5% DMSO-treated sugar (10% glucose solution) *ad libitum* for 24 hours right before an infectious blood meal. Oocyst intensity and prevalence of infection is measured day 7 pIBM. The experimental design from Figure 6 is used.

It was, however, unclear if this effect can be observed at lower concentrations of ATQ. Therefore, an ATQ dilution experiment was conducted, where female *A. gambiae* were fed 100μM ATQ + 0.5% DMSO, 10μM ATQ + 0.5% DMSO, and 1μM ATQ + 0.5% DMSO, in 10% glucose solutions *ad libitum* for 24 hours before receiving an

infectious blood meal. ATQ dilutions resulted in a dose-dependent inhibition of *P. falciparum* development in *A. gambiae* (Figure 8). The prevalence of infection decreased as the concentration of ATQ in the sugar meal increased. At 100μM ATQ, 10μM ATQ, and 1μM ATQ, the prevalence of infection for these exposure groups were 9.7%, 34.2%, and 59.3%, respectively (prevalence: two-sided Fisher's Exact Test with Bonferroni correction, 100μM ATQ, $n = 199$, $p < 0.0001$; 10μM ATQ, $n = 175$, $p < 0.0001$; 1μM ATQ, $n = 182$, $p = 0.1065$). Despite this significant dose-dependent decrease in the prevalence of infection in the ATQ-exposed groups, there was no significant difference in the oocyst intensity between the control group and the ATQ-treated groups (intensity: Kruskal-Wallis test for multiple comparisons, 100μM ATQ, $n = 199$, $p > 0.9999$; 10μM ATQ, $n = 175$, $p > 0.9999$; 1μM ATQ, $n = 182$, $p > 0.9999$). The results from the pre-infection ATQ sugar feeding showed the potential for using ATQ in ATSBs for malaria control.

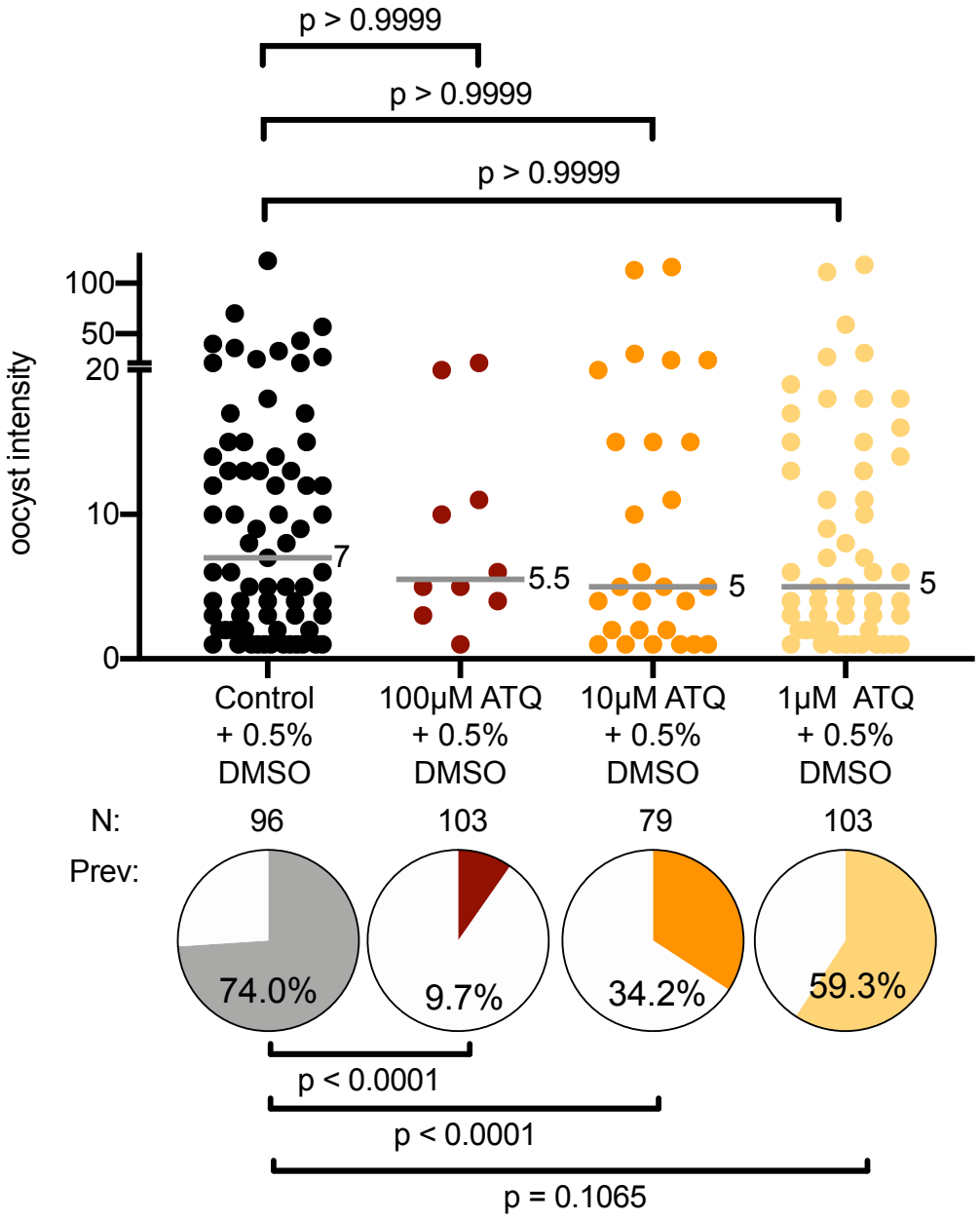

Figure 8 Dilution series for ATQ sugar feeding assay. Results show the oocyst intensity and prevalence of infection of *P. falciparum* after *A. gambiae* females are exposed to 100μM ATQ + 0.5% DMSO, 10μM ATQ + 0.5% DMSO, and 1μM + 0.5% DMSO, treated sugar (10% glucose solution) *ad libitum* for 24 hours right before an infectious blood meal. Oocyst intensity and prevalence of infection was measured day 7 pIBM. The experimental design from Figure 6 is used.

II. ATQ exposure via sugar feeding after an infectious blood meal showed significant inhibitory effects on the transmission of *P. falciparum* in *A. gambiae*.

A mosquito continues sugar feeding after it takes a blood meal. Therefore, it was important to assess the effects of ATQ on post-infection parasite development. Females were starved for 24 hours before they were provided an NF54, *P. falciparum* blood meal, and 2 days pIBM, they were provided a continuous exposure to ATQ-treated sugar to determine whether ATQ-treated sugar feeding had an effect after a mosquito was infected (Figure 9). The oocyst intensity, prevalence, and oocyst size were measured on days 7, 10, and 14 pIBM, and sporozoites were measured at day 14 pIBM. Based on these results, post-infection exposure to ATQ-treated sugars was carried out to determine the efficacy of ATQ in inhibiting *P. falciparum* in already-infected mosquitoes.

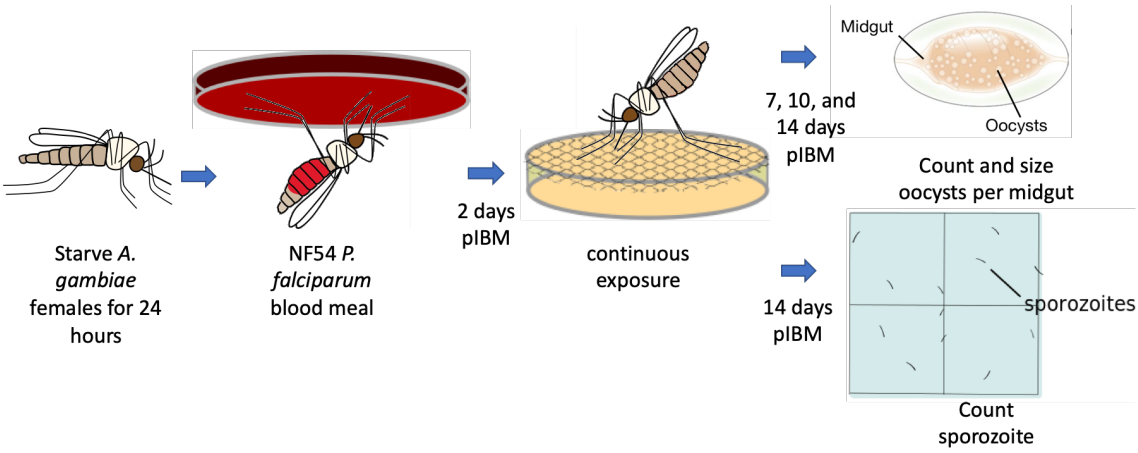

Figure 9 Post-infection sugar-feeding assay: Female *A. gambiae* mosquitoes were starved for 24 hours before given an infectious blood meal. Two days pIBM, *A. gambiae* females were given a continuous exposure of 100µM ATQ in 10% w/v glucose *ad libitum*. At days 7, 10, and 14, the prevalence and intensity of *P. falciparum* infection was measured. At day 14, the sporozoites per mosquito was counted.

Female *A. gambiae* mosquitoes were infected with *P. falciparum* and then continuously exposed to 100µM ATQ + 0.05% DMSO in 10% glucose concentration *ad libitum* two days after the infectious blood meal (Figure 9). The oocyst intensity, prevalence of infection, and average oocyst size per mosquito were observed at 7, 10, and 14 days after infection (Figure 10 and Figure 11). No significant differences were observed in oocyst intensity on any of the days (intensity: Kruskal-Wallis test for multiple comparisons; day 7, n = 100, p > 0.9999; day 10, n = 84, p = 0.3229; day 14, n = 75, p > 0.9999, d7:d10 controls, n = 97, p > 0.9999, d10:d14 controls, n = 89, p > 0.9999, d7:d14 controls, n = 90, p > 0.9999). A significant inhibition of the prevalence of infection was instead observed between the control and ATQ-treated groups at day 7, while no difference was observed at day 10 nor at day 14 (prevalence: two-sided chi-

squared test; day 7, n = 100, p = 0.0060; day 10, n = 84, p > 0.9999; day 14, n = 75, p = 0.1100, d7:d10 controls, n = 97, p > 0.9999, d10:d14 controls, n = 89, p > 0.9999, d7:d14 controls, n = 90, p > 0.9999).

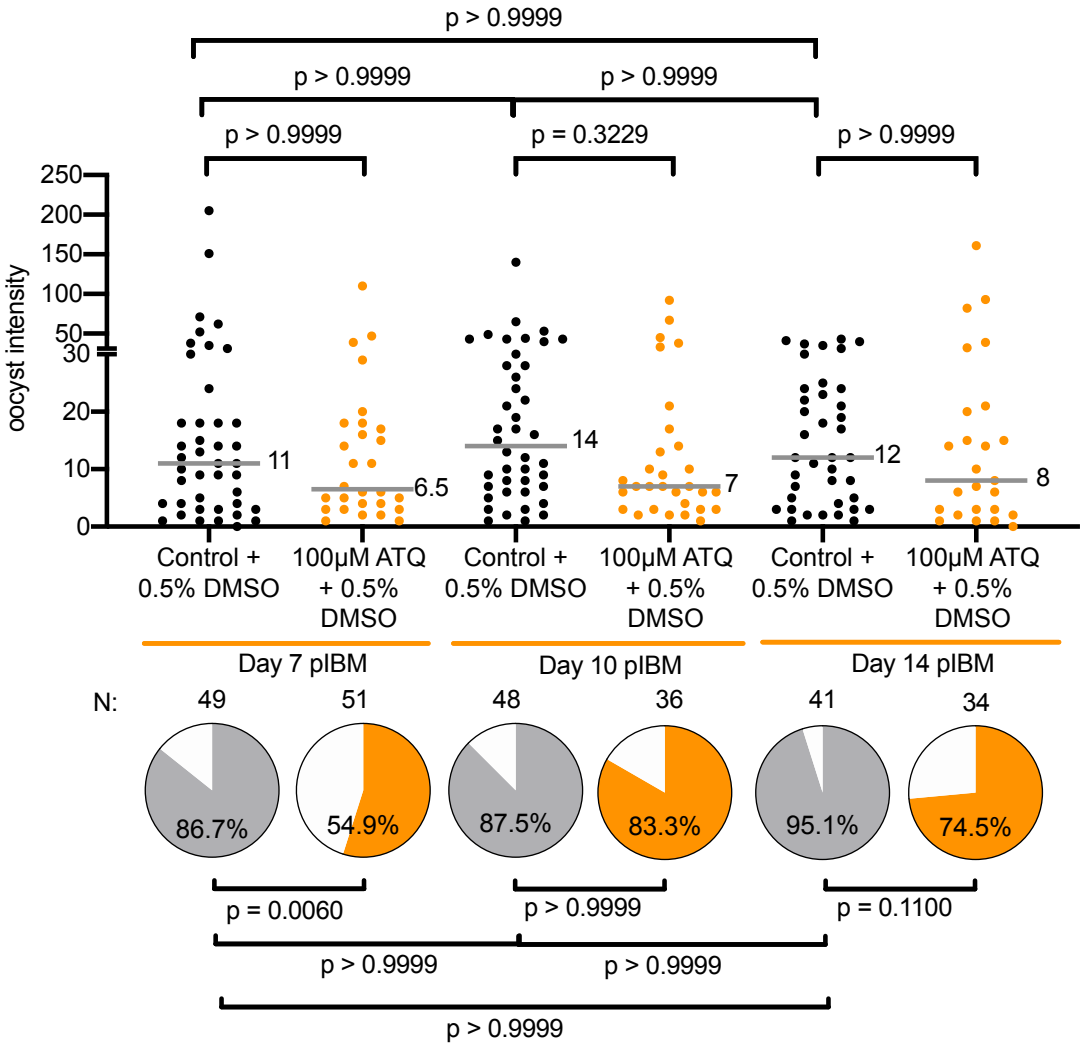

Figure 10 Results show the oocyst intensity and prevalence of infection of *P. falciparum* after *A. gambiae* mosquitoes were continuously exposed to 100μM ATQ + 0.5% DMSO-treated sugar (10% glucose solution) *ad libitum* two days after their infectious blood meal. Oocyst intensity and prevalence of infection were collected on day 7, day 10, and day 14 pIBM. The experimental design from Figure 9 is used.

There was however a notable difference in the size of the oocysts, which suggested an inhibitory effect of ATQ on the development of *P. falciparum* in the mosquito midgut when fed ATQ-treated sugar continuously pIBM (Figure 11). At day 7, the mean of the average oocyst size per mosquito was 72 μm^2 in the 100µM ATQ group as compared to374 μm^2 in the control group. At day 10 and day 14, the average oocyst size per mosquito in the control and ATQ groups both increased in size; however, significant differences were still observed between the groups. At day 10, the mean of the average oocyst size per mosquito was 165 μm^2 in the 100µM ATQ group, which was a significant decrease from the control group with mean of the average oocyst size per mosquito of 843 μm^2. Day 14 increased to 337μm^2 for the ATQ treated group while the control group was 1423μm^2 mean of the average oocyst size per mosquito. (Intensity: unpaired T-test; day 7, $n = 69$, $p < 0.0001$; day 10, $n = 70$, $p < 0.0001$; day 14, $n = 61$, $p < 0.0001$.) The size of the oocysts in the ATQ-treated group at day 14 suggested that the oocysts have not matured enough to produce sporozoites.

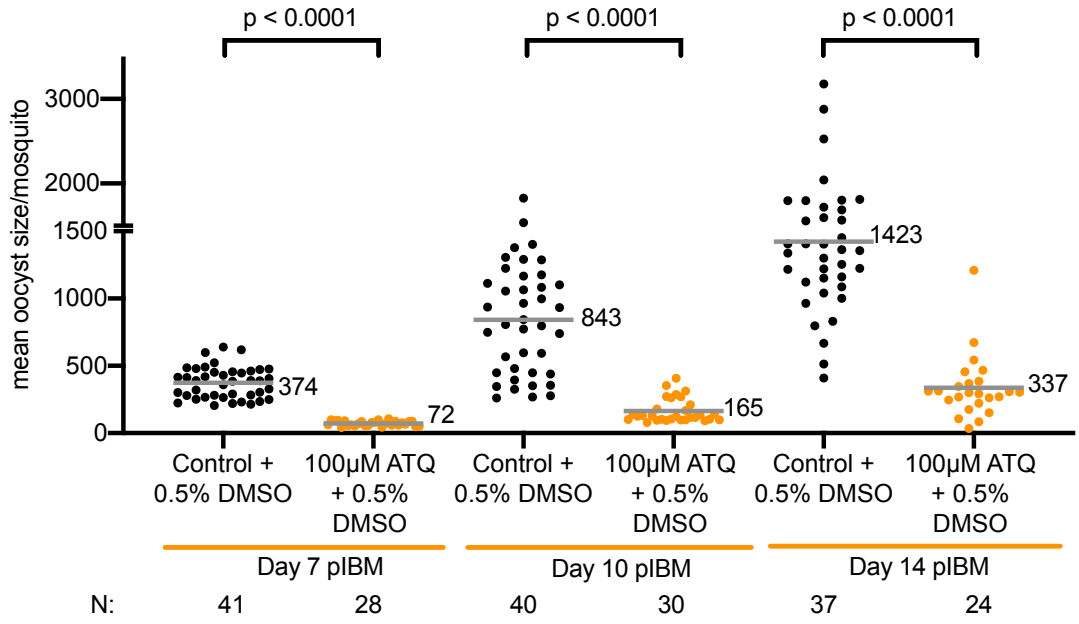

Figure 11 Results show the oocyst size of *P. falciparum* after *A. gambiae* mosquitoes were continuously exposed to 100µM ATQ + 0.5% DMSO-treated sugar (10% glucose solution*) ad libitum* two days after their infectious blood meal. Oocyst size were measured on day 7, day 10, and day 14 pIBM. The experimental design from Figure 9 is used.

To confirm that the oocysts at day 14 after continuous ATQ-sugar exposure were not mature enough to produce sporozoites, the sporozoites were extracted from the salivary glands and counted. The ATQ-treated group had no sporozoites (Figure 12), while in the control group the median number of sporozoites per mosquito was 8,400 (prevalence: two-sided Fisher's Exact Test, n = 86, $p < 0.0001$). The complete inhibition of sporozoite development in ATQ-treated mosquitoes indicated that the mosquitoes were not infectious at day 14, unlike the control mosquitoes.

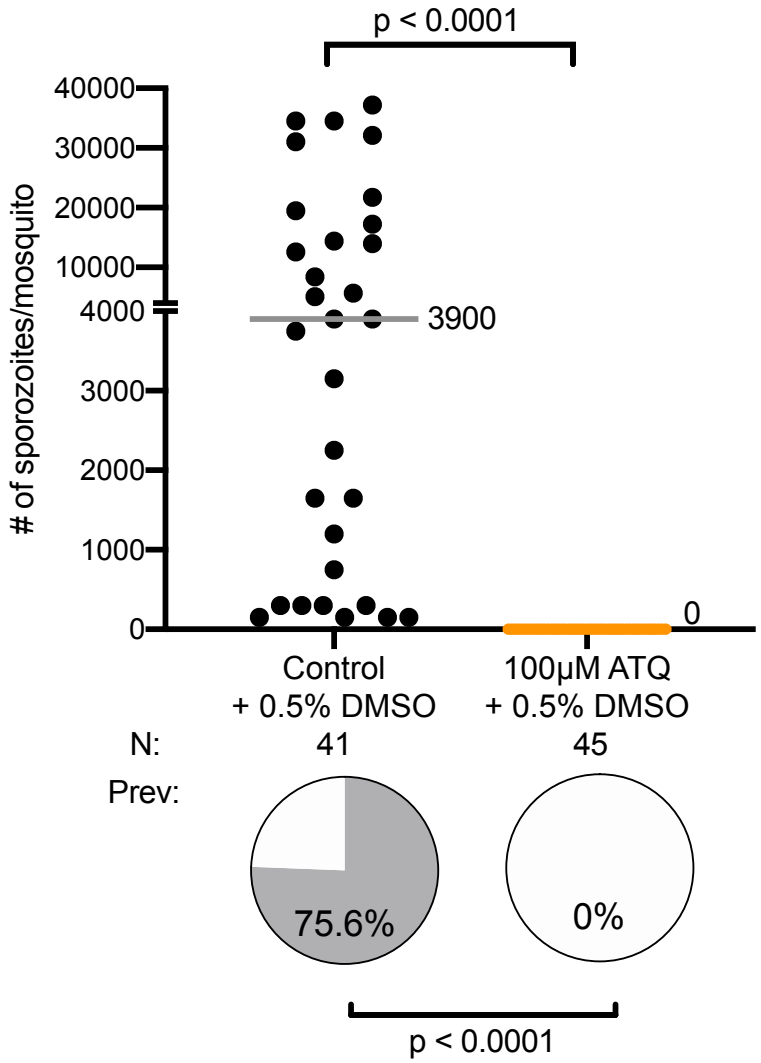

Figure 12 Results show the number of *P. falciparum* sporozoites after *A. gambiae* mosquitoes were continuously exposed to 100μM ATQ + 0.5% DMSO-treated sugar (10% glucose solution) *ad libitum* two days after their infectious blood meal. The number of sporozoites are taken from the mosquito salivary glands on day 14 pIBM. The experimental design from Figure 9 is used.

III. HYD exposure through sugar feeding caused a dose-dependent decrease in the survival of *A. gambiae* females.

It is known that HYD has insecticidal effects on other arthropods and is commonly used in ants and cockroaches (Decio et al., 2013). However, insecticidal effects of HYD has been tested against *Anopheles* and *Aedes* mosquitoes and was shown to have no effect by tarsal contact. Similarly to ATQ, HYD can also be used as a vector control tool and might have potential antimalarial effects through sugar feeding as it was seen to have through tarsal contact, Figure 5 (Paton et al., 2019). Females are starved for 24 hours prior to exposure to HYD for 24 hours (Figure 13). Dead mosquitoes are counted and removed daily. This survival assay was carried out to see the effects of HYD on mosquito survival and to determine a suitable sub-lethal concentration of HYD to use for infection-inhibition experiments.

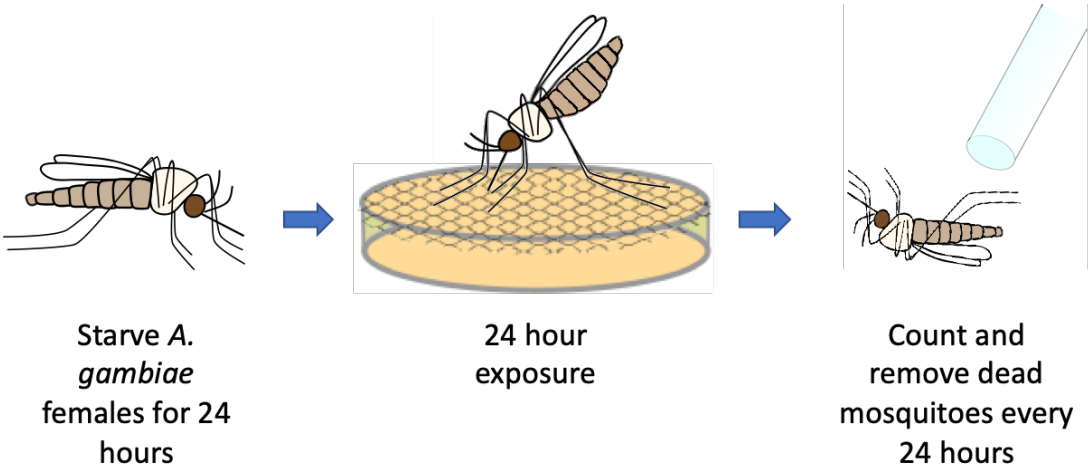

Figure 13 HYD survival assay: *A. gambiae* females are starved for 24 hours before they are fed a HYD-treated sugar meal for 24 hours. The number of dead mosquitoes were counted and removed every 24 hours.

HYD at 100μM, 10μM, and 1μM were compared to three control groups—a standard control with 10% glucose solution and 0.5% DMSO, a water only control, and a control with nothing in the cage (Figure 14). Survival of the mosquitoes was measured by counting the number of dead mosquitoes every 24 hours after the removal of the exposures. At 100μM HYD, the mosquitoes had the most significant change in survival. By day 7 of the survival assay, all of the mosquitoes have died in the 100μM HYD exposure. As seen in Table 1, the risk ratio for 100μM HYD as compared to all the other treatments are all greater than 1, signifying that the mosquitoes given 100μM HYD had a higher risk of dying than the other groups (two-sided log-rank Mantel-Cox, 100μM:Nothing, $n = 80$, $RR = 3.51$, $p < 0.0001$; 100μM:Water, $n = 80$, $RR = 6.80$, $p < 0.0001$; 100μM:Sugar, $n = 80$, $RR = 7.59$, $p < 0.0001$; 100μM: 1μM, $n = 80$, $RR = 8.00$, $p < 0.0001$; 100μM: 10μM, $n = 80$, $RR = 5.33$, $p < 0.0001$). While the nothing control also had significant death at the beginning of the survival assay, there is statistically significant evidence that 100μM-HYD treated mosquitoes are 3.51 times more likely to die than mosquitoes given nothing. The 10μM HYD exposure had a milder decrease in survival, with the deaths plateauing at day 15 of the assay. Nonetheless, the risk of dying with a 10μM HYD exposure was not significantly greater than mosquitoes given a water control and a sugar control (two-sided log-ranked Mantel-Cox, 10μM:Water, $n = 80$, $RR = 1.28$, $p = 0.2791$; 10μM:Sugar, $n = 80$, $RR = 1.42$, $p = 0.1163$). When the mosquitoes were exposed to 1μM HYD, there was no significant decrease in survival as compared to the water and sugar controls (1μM: Water, $n = 80$, $RR = 0.85$, $p = 0.4642$; 1μM: Sugar, $n = 80$, $RR = 0.95$, $p = 0.8115$).

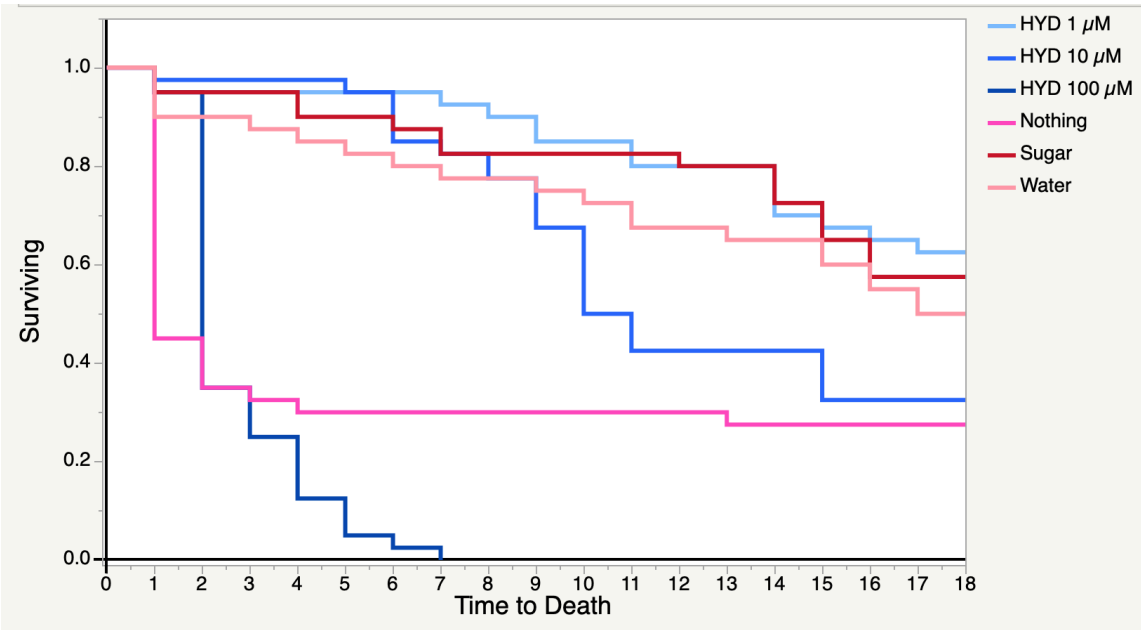

Figure 14 A dilution series survival assay was performed by exposing *A. gambiae* females to a dilution of HYD (100µM, 10µM, and 1µM) + 0.5% DMSO in 10% glucose solution *ad libitum* for 24 hours. After 24 hours, 10% glucose solution without treatment, *ad libitum*, was given to the mosquitoes for the duration of the experiment. Dead mosquitoes were counted and removed every 24 hours. The experimental design from Figure 13 is used.

Treatment 1	Treatment 2	Risk Ratio[1]	*P*
Nothing	HYD 100 µM	0.2852471	<.0001*
Water	HYD 100 µM	0.1471669	<.0001*
Sugar	HYD 100 µM	0.1317773	<.0001*
HYD 1 µM	Water	0.8489229	0.4642
HYD 1 µM	Sugar	0.9480638	0.8115
HYD 10 µM	Water	1.2751832	0.2791
HYD 10 µM	Sugar	1.4241045	0.1163
HYD 100 µM	Nothing	3.5057323	<.0001*
HYD 100 µM	Water	6.7950082	<.0001*
HYD 100 µM	Sugar	7.5885581	<.0001*
HYD 100 µM	HYD 1 µM	8.0042698	<.0001*
HYD 100 µM	HYD 10 µM	5.3286526	<.0001*

Table 1 The experimental design from Figure 13 is used. The survival of the two treatments are compared, and the risk ratio and significance are given in this graph. Risk ratio greater than 1 signifies that Treatment 1 has a greater likelihood to die by a multiple of the risk ratio than Treatment 2. (See the rest of this in the Appendix Table 2.)

IV. HYD exposure through sugar feeding had a significant effect on the prevalence of *P. falciparum* infection.

It was seen, through the survival assay, that 10μM decreased *A. gambiae* survival but the effect was not significant (Table 1). Therefore, 10μM HYD was used to observe the drug's ability to work as an antimalarial. The mosquitoes were fed 10μM HYD + 0.5% DMSO in 10% glucose *ad libitum* for 24 hours right before an infectious blood meal, and the oocysts were counted at day 7 post infection (Figure 6). At 10μM HYD, there was no significant effect of HYD on oocyst intensity, with the median oocysts per midgut at 7 for control group and 11 for the HYD-treated group. There was, however, a statistically significant inhibition of *P. falciparum* prevalence of infections in *A. gambiae* when treated with 10μM HYD (Figure 15). There was a 16.1% decrease in the prevalence of infection between the control group and the HYD-treated group (prevalence: two-sided Fisher's Exact Test, $n = 134$, $p = 0.0231$; intensity: two-sided Mann-Whitney U test, $n = 134$, $p = 0.1716$).

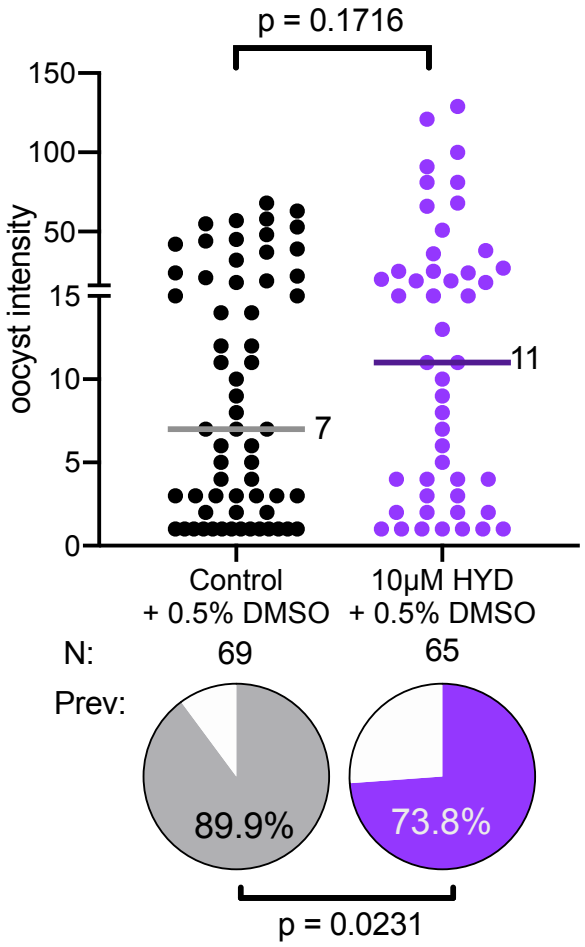

Figure 15 Prevalence of infection and oocyst intensity are shown. *A. gambiae* females were exposed to 10μM HYD + 0.5% DMSO in 10% glucose solution *ad libitum* for 24 hours right before an infectious blood meal. The *P. falciparum* prevalence of infection and oocyst intensity were observed on day 7 pIBM. The experimental design from Figure 6 is used.

V. MET administered through tarsal contact did not affect *P. falciparum* infection in *A. gambiae* mosquitoes.

Previous data has shown that MET, when topically applied on the thorax of *A. gambiae* mosquitoes, decreased the prevalence of *P. falciparum* infections (Childs et al., 2016). It was unclear how effective MET was through tarsal contact, a more likely way for mosquitoes to take up compounds in field-like settings. Female *A. gambiae* mosquitoes were exposed to 1mmol MET coating a glass dish for an hour before an infectious blood meal, and the number of oocysts per midgut were measured 7 days post infection (Figure 16). Egg development 7 days pIBM was assessed as well (Figure18).

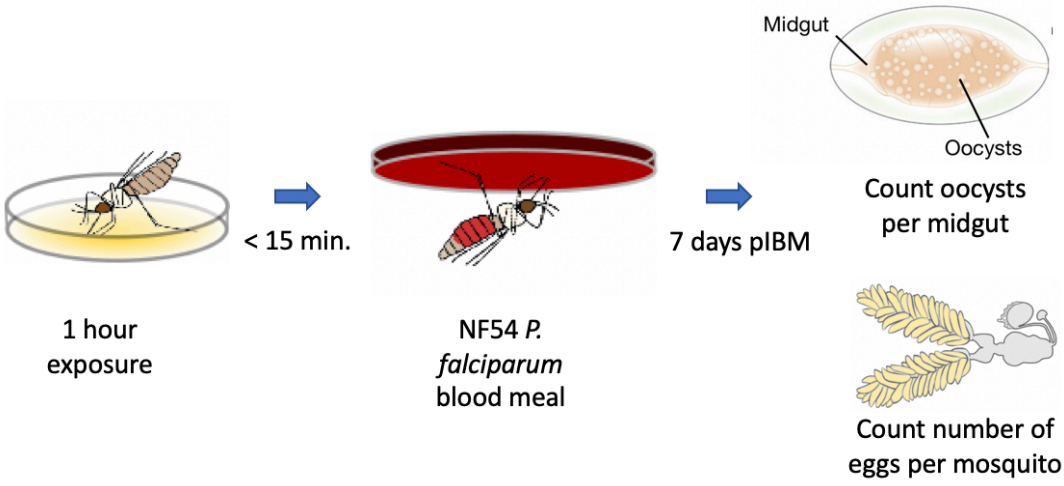

Figure 16 Virgin female *A. gambiae* mosquitoes were exposed to 1 mmol/m^2 MET for an hour right before being fed an infectious blood meal. Seven days pIBM, the prevalence and intensity of *P. falciparum* infection and egg development are assessed.

Exposing virgin female *A. gambiae* mosquitoes to 1 mmol MET showed little to no significant effect on the prevalence and intensity of infection on mosquito midguts

taken on day 7 pIBM (Figure 17). While there is a decrease between the median of the control group and of the 1mmol MET group—31 oocysts per midgut and 17 oocysts per midgut, respectively— the decrease was not statistically significant. There is also an insignificant but visible increase in the oocyst intensity in the RME + MET group, where the median oocyst intensity is 42 compared to 34 in RME only. (prevalence: two-sided Fisher's Exact Test with Bonferroni correction; control: MET, $n = 94$, $p > 0.9999$; RME: MET + RME, $n = 102$, $p > 0.1374$, control:RME, $n = 95$, $p > 0.9999$; intensity: Kruskal-Wallis test with multiple comparisons; control:MET, $n = 94$, $p = 0.9159$, RME: MET+RME, $n = 102$, $p > 0.9999$, control:RME, $n = 95$, $p = 0.7909$).

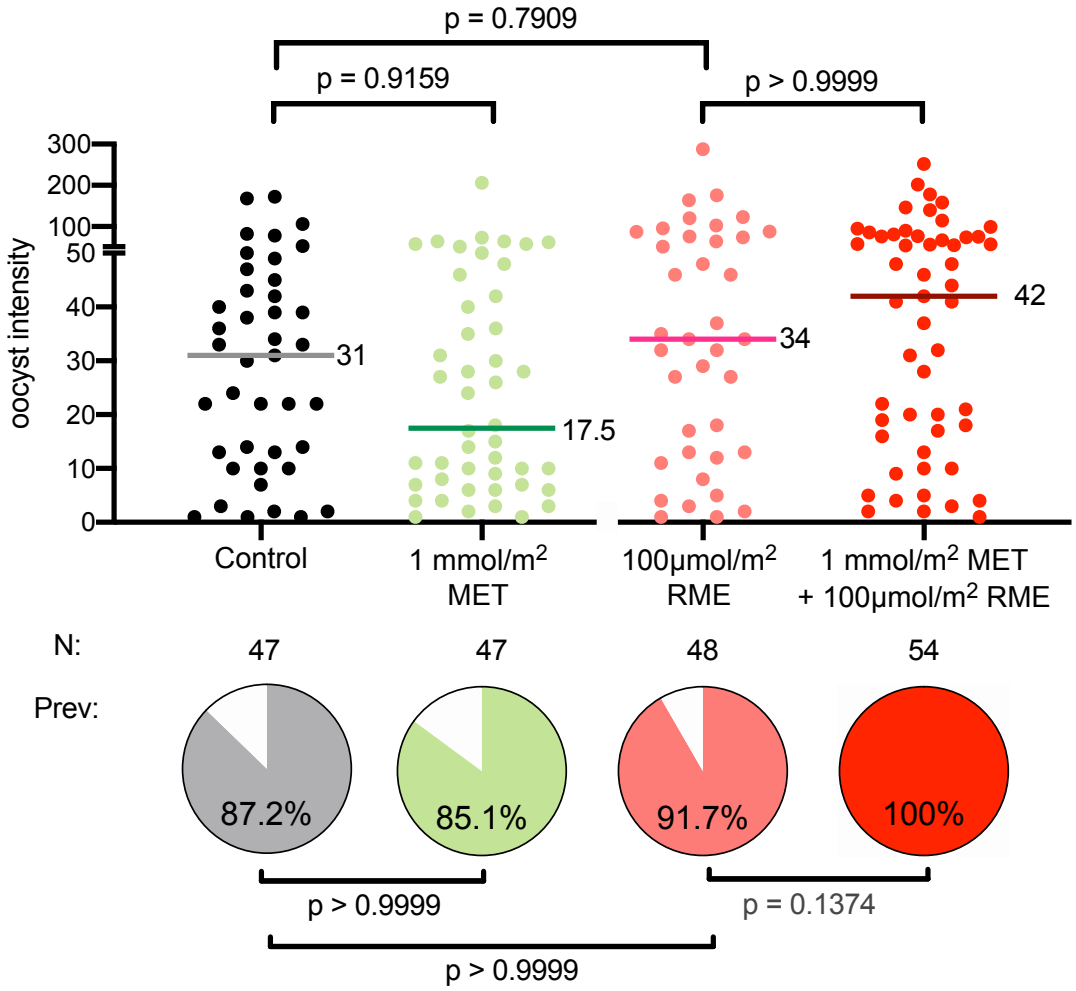

Figure 17 Prevalence of infection and oocyst intensity are shown. *A. gambiae* females were exposed to a control, 1mmol MET, 100µmol RME, or 1mmol MET + 100µmol RME on a glass dish an hour right before an infectious blood meal. The *P. falciparum* prevalence of infection and oocyst intensity were observed on day 7 pIBM. The experimental design from Figure 16 is used.

VI. MET administered through tarsal contact had no effect on egg development in *A. gambiae* mosquitoes.

Along with looking at oocyst intensity after 1 mmol MET exposure, egg development was also observed from this group of mosquitoes. As mentioned, MET is an insect growth regulator with known effects to decrease egg development in *A. gambiae* in a dose-dependent manner when topically applied on to the thorax (Childs et al., 2016). Virgin female *A. gambiae* mosquitoes were used because females typically cannot oviposit without mating. When exposing *A. gambiae* to MET through tarsal contact, there were no significant differences when comparing the egg development in the control to 1mmol MET and in 100 µmol RME to 100 µmol RME + 1 mmol MET, as seen in Figure 18 (intensity: ordinary one-way ANOVA with multiple comparisons; control:MET, n = 105, p = 0.4061; RME:MET+RME, n = 102, p = 0.9709, control:RME, n = 99, p = 0.9509). While there were no significant differences in the decrease between the control and MET exposure groups, there seemed to be a consistent but insignificant decrease in the number of eggs developed per mosquito in the MET group when compared to the control group (Figure 18).

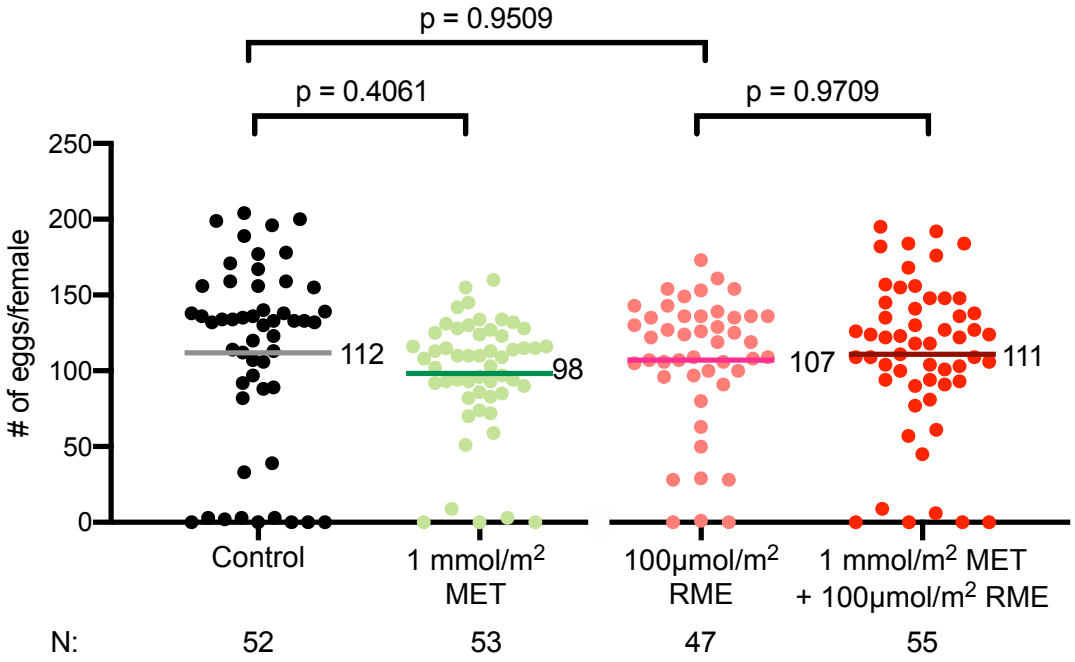

Figure 18 Results show the number of eggs in each virgin female *A. gambiae*. Females were exposed to a control, 1mmol MET, 100µmol RME, or 1mmol MET + 100µmol RME on a glass dish 1-hour right before an infectious blood meal. The number of eggs per female was observed on day 7 pIBM. The experimental design from Figure 16 is used.

This experiment was followed up with an oviposition assay where the same exposure groups were measured for the number of eggs laid and prevalence of egg laying amongst exposed, virgin female *A. gambiae*. After a 1-hour exposure, female mosquitoes are given a noninfectious blood meal. At 3 days pIBM, the number of eggs developed and laid were counted (Figure 19).

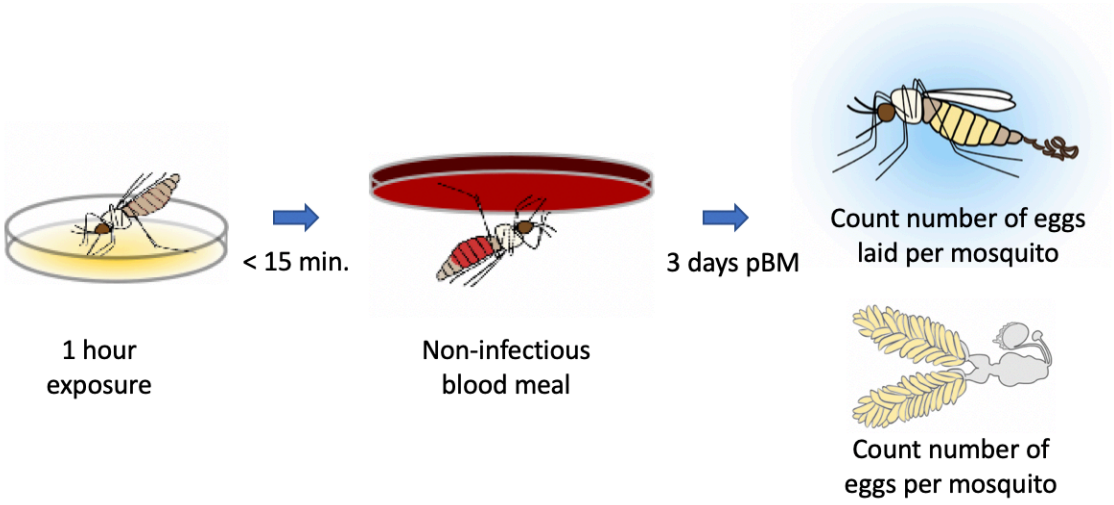

Figure 19 Virgin female *A. gambiae* females are exposed to MET for an hour before a non-infectious blood meal. Virgin females were used because female mosquitoes typically cannot oviposit without mating. Three days pBM, the prevalence of oviposition, number of eggs laid per female, and number of eggs per mosquito is counted.

Under normal circumstances, females must be mated and take a blood meal in order to lay eggs; however, it has been shown that MET can induce egg laying in blood fed virgin female mosquitoes when administered through topical application on the thorax (Childs et al., 2016). In the oviposition assay (Figure 20), there were no significant differences between the control groups and the MET exposure groups in terms of the number of eggs laid nor in terms of the prevalence of virgin females that laid their eggs (intensity: ordinary one-way ANOVA with multiple comparisons; control:MET, n = 126, p = 0.9947; RME:MET+RME, n = 137, p = 0.9787, control:RME, n = 130, p = 0.8930; prevalence: two-sided Fisher's Exact Test with Bonferroni corrections; control:MET, n = 126, p > 0.9999; RME:MET+RME, n = 137, p > 0.9999, control:RME, n = 130, p = 0.9933).

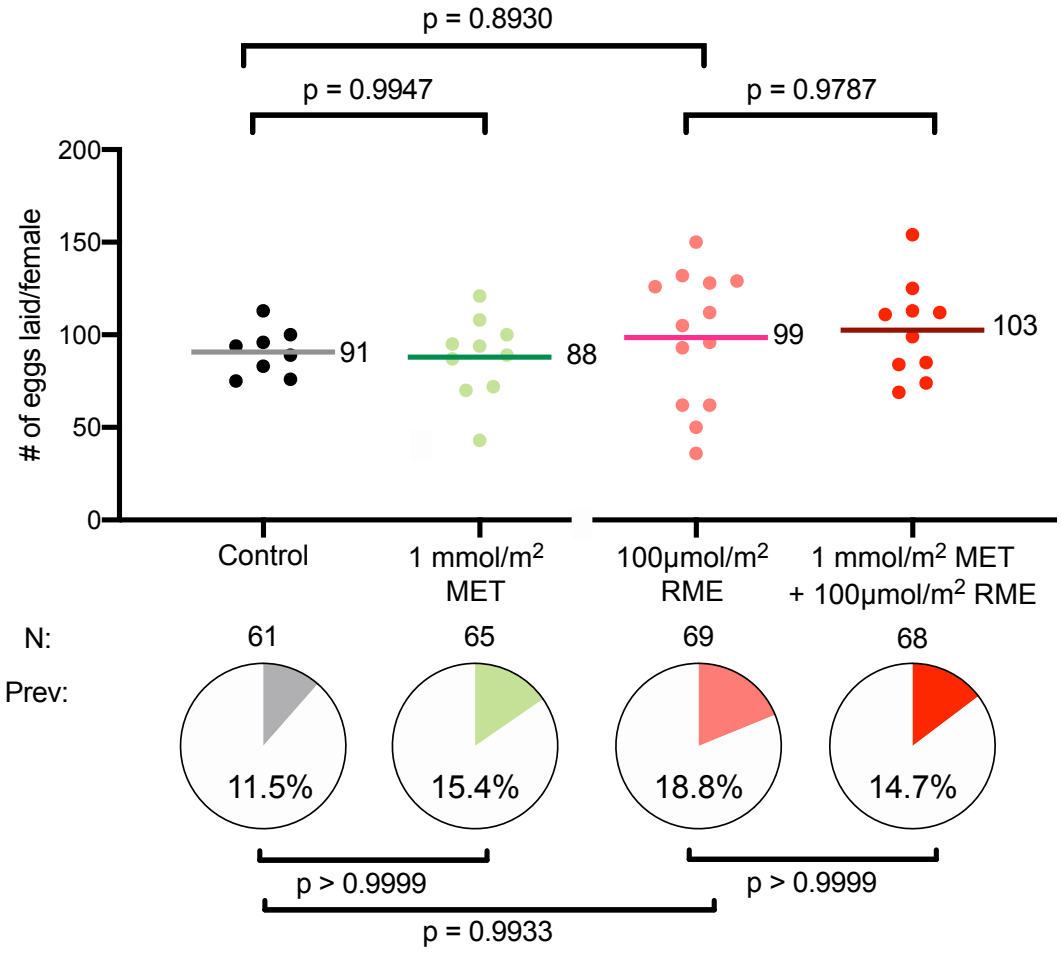

Figure 20 The number of eggs laid per virgin female *A. gambiae* and the prevalence of females that laid their eggs is shown above. Female *A. gambiae* mosquitoes were exposed to a control, 1mmol MET, 100µmol RME, or 1mmol MET + 100µmol RME on a glass dish 1-hour right before a regular, non-infectious blood meal, and eggs were counted 3 days after the blood meal. The experimental design from Figure 19 is used.

VII. MET administered through sugar feeding significantly decreased egg development in virgin female *A. gambiae*.

To establish a more effective method of administering MET, mosquitoes were starved for 24 hours and then fed a 10% glucose solution mixed with a 100 μM MET and 0.5% DMSO solution for 24 hours before their noninfectious blood meal (Figure 19). As expected, there was a significant decrease, as seen in Figure 21, in the number of eggs developed in each virgin female mosquito, where a mean of 125 eggs per female was observed in the controls and a mean of 107 eggs per female was observed in the treated (intensity: unpaired t-test, $n = 165$, $p = 0.0010$). This data supported the results from a previous MET study that MET decreased the egg development in female mosquitoes (Childs et al., 2016).

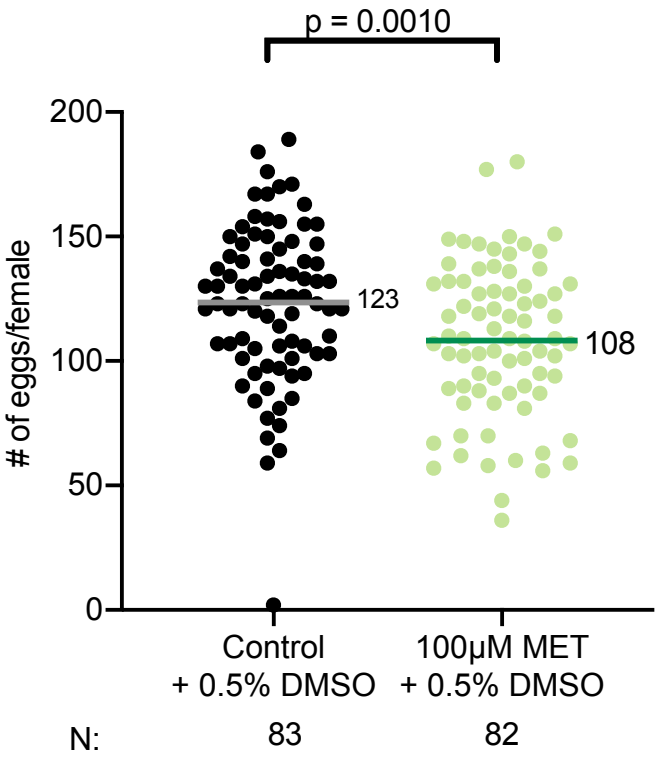

Figure 21 The number of eggs developed per virgin female *A. gambiae* is shown above. Females were exposed to 100µM MET + 0.5% DMSO in 10% glucose solution *ad libitum* for 24 hours right before a non-infectious blood meal. Egg development is counted 3 days after the blood meal. The experimental design from Figure 6 is used.

VIII. MET administered through tarsal contact showed no significant effects on 20E levels 24 hours after a blood meal.

In order to understand how MET is affecting 20E signaling in female mosquitoes, a 20E enzyme immunoassay (EIA) was used to detect 20E levels. Females were exposed to 100µmol RME only and 100µmol RME + 1 mmol MET at 24 hours before a blood meal and at 0 hours before a blood meal for 1 hour via tarsal contact (Figure 22). The 20E levels were detected 24 hours after a blood meal using a 20E enzyme immunoassay.

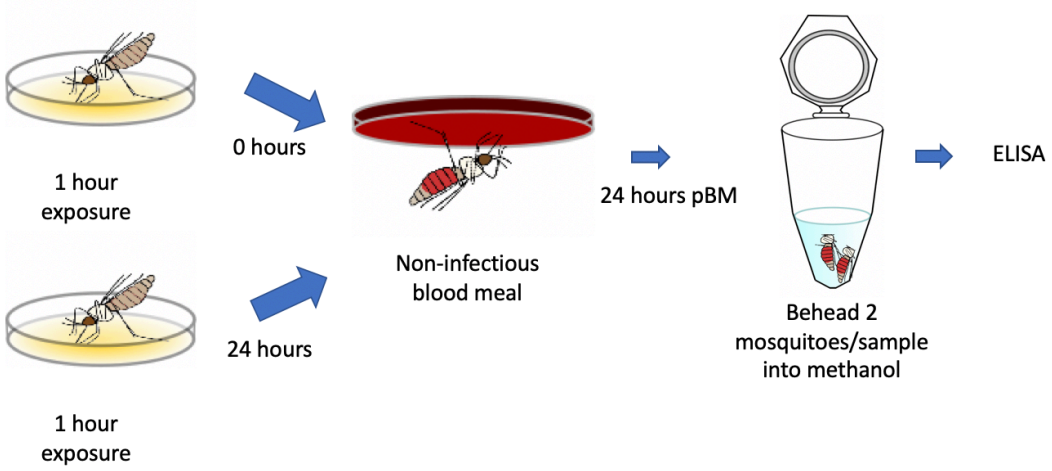

Figure 22 Virgin female *A. gambiae* mosquitoes were exposed to 1mmol/m^2 MET through tarsal contact for an hour either right before a non-infectious blood meal or 24 hours before a non-infectious blood meal. Twenty-four hours after the blood meal, blood-fed females were beheaded and stored in methanol, in preparation for an ELISA to detect 20E levels.

When the virgin female mosquitoes were exposed 24 hours before a blood meal, there was no significant difference in 20E levels between the control, RME only, group and the exposure, RME plus MET, group. When the virgin female mosquitoes were exposed 0 hours before a blood meal, there was no statistically significant difference in 20E levels between the control and treated groups (Figure 23). However, there is a noticeable but insignificant decrease in the RME+MET groups in both the 24 hours before blood feed and 0 hours before blood feed groups (20E detection intensity: one-way ordinary ANOVA with multiple comparisons; 0 hours, $n = 36$, $p = 0.4035$; 24 hours, $n = 34$, $p = 0.9292$, RME (0 hours):RME (24 hours), $n = 35$, $p = 0.1294$).

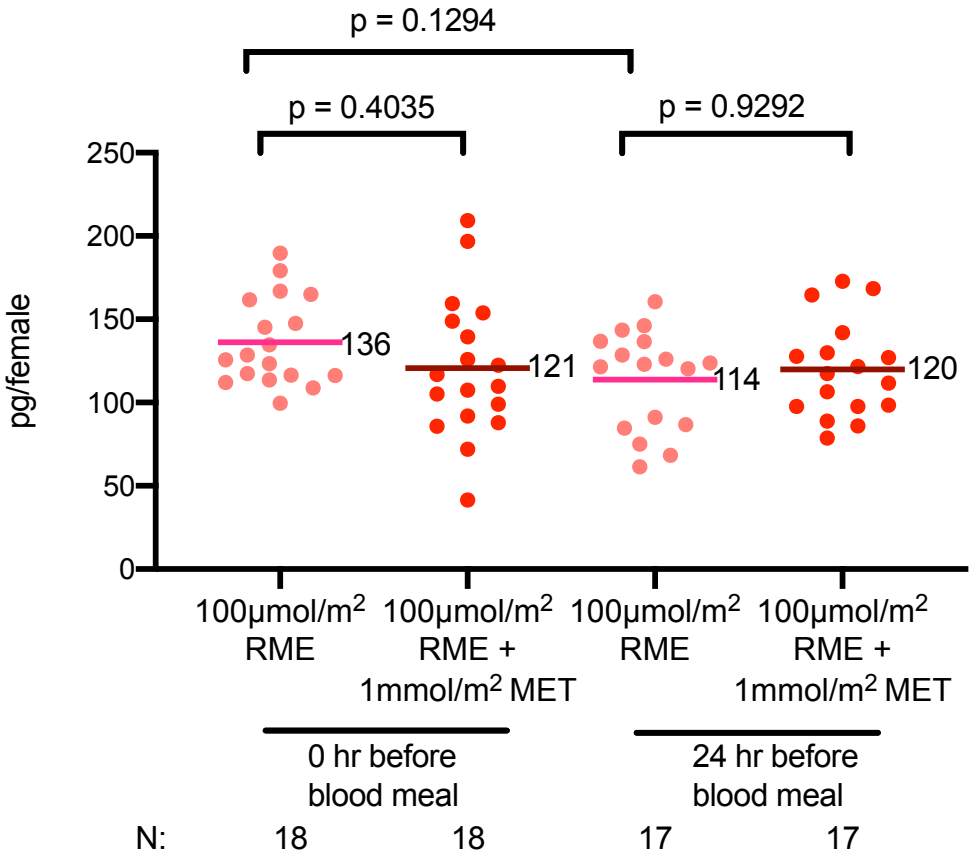

Figure 23 An ELISA was performed to detect the levels of 20E in *A. gambiae* females exposed to 1mmol MET + 100µmol RME or 100µmol RME only either 0 hours before a blood meal or 24 hours before a blood meal. 20E levels are measured at 24 hours after the blood meal, when 20E levels are at its peak. The experimental design from Figure 22 is used.

DISCUSSION

P. falciparum development has been successfully arrested by exposing *A. gambiae* females to ATQ-treated sugar meals either before or after an infectious blood meal. Paton, et al., previously established that there was a dose-dependent effect of ATQ exposure on the prevalence and intensity of *P. falciparum* infections when ATQ was administered before an infectious blood meal through tarsal contact. There was an observed arrest of parasite development at the zygote stage, so oocysts did not develop (Paton et al., 2019). The pre-infection sugar feeding assays, as seen in Figure 8, also showed a dose-dependent decrease in the prevalence of *P. falciparum* infection, but this dose-dependent effect was not reflected in the oocyst intensity. While these results indicate that pre-infection ATQ-treated sugar feeding can successfully arrest parasite development, further research must be done to determine the stage at which pre-infection ATQ-treated sugar feeding blocks parasite development. An immunofluorescent assay using the parasite-specific antibody, anti-Pfs25, can be done 18-24 hours after a *P. falciparum* blood meal on the midgut lumen, where the bloodmeal is held. The time 18-24 hours after a blood meal is consistent with when ookinetes should have already formed, as seen above in Figure 3. Pfs25 surface proteins are found on developing zygotes and ookinetes in the midgut lumen (Barr et al., 1991; Vermeulen et al., 1985). It is hypothesized that, like the pre-infection ATQ-treated tarsal contact exposures, pre-infection ATQ-treated sugar feeding will also block parasite development predominantly at the zygote stage. Determining the stage at which pre-infection ATQ-treated sugar feeding halts *P. falciparum* infections in *A. gambiae* can help develop a better picture of

the similarities and differences between pre-infection sugar feeding and pre-infection tarsal contact exposures. Despite having no effect on oocyst intensity, the use of ATQ-treated sugar meals can greatly reduce prevalence of malaria infections in mosquito populations. The inability for parasites to survive at 100μM ATQ can decrease vector capacity, leading to decreased transmission of malaria to humans.

As seen in Figure 7, it is clear that 100μM ATQ can completely suppress oocyst development in the midgut; however, as seen in Figure 8, complete suppression does not always occur across multiple replicates. It is likely that the 10 infected mosquitoes in the 100μM ATQ-treated group were individual mosquitoes that did not imbibe the ATQ-treated sugar meals or did not imbibe as much sugar. This could be due to environmental differences that provided mosquitoes with enough moisture to forgo drinking treated sugars or due to individual differences where some did not need to imbibe sugar as often for energy metabolism. Further research must be conducted to determine whether the incomplete suppression is a behavioral effect, where some mosquitoes are not feeding on the ATQ-treated sugar, or some form of metabolic or target-site resistance against ATQ. To determine if this is a feeding behavior effect, the 10% glucose solutions can be mixed with food dye. Dyed sugar can be observed, by eye, in the mosquito crop, as mosquitoes are somewhat translucent. If all the mosquitoes feed on the ATQ-treated sugar during a 24-hour period across multiple replicates, then it can be assumed that factors other than behavior, such as ATQ resistance, are at play. If some of the mosquitoes do not feed on the ATQ-treated sugar during a 24-hour period across multiple replicates, it can be said that sugar feeding behavior plays a role in the incomplete suppression of oocyst

development. However, to confirm that the behavioral effects are not mixed with effects of ATQ resistance, *A. gambiae* females that did not feed on the sugar meal, as seen through the presence of dye, should be removed prior to an infectious blood meal. Repellency to ATQ-treated sugar meals in lab settings would only be exaggerated in field settings, where mosquitoes have access to other sources of sugar meals. While attractants were not added into ATQ-treated sugar meals in the experiment, it is important to replicate this experiment in field-like settings with attractants and other sources of sugar meals to determine if mosquitoes will choose ATQ-treated ATSBs over other options. ATQ, nonetheless, has strong evidence of success in reducing malaria transmission and should be added into the developing arsenal of malaria control tools.

The pre-infection ATQ-treated sugar feeding dilution series assay produces an all-or-nothing response in prevalence of infection and oocyst intensity, as seen in Figure 8. While there were significant decreases in the prevalence of infection when given increasing concentrations of ATQ-treated sugar meals, this observation did not carry through to the oocyst intensity, where the number of oocysts per midgut showed no significant changes across the different groups, including the control group. Suppression was occurring in a dose-dependent manner, but in mosquitoes where complete suppression did not occur, there was no effect on *P. falciparum* infection. Despite explaining the incomplete suppression at 100μM ATQ with potentially non-feeding mosquitoes, the increasing prevalence of infection in the 10μM and 1μM ATQ treatment groups should be due to the decreasing concentration of ATQ rather than to repellency to ATQ-treated sugar meals. It is unlikely that more mosquitoes were repelled from feeding

on lower concentrations of ATQ. To rule out behavioral repellency to ATQ-treated sugar meals, an ATQ dilution series with dye mixed into 10% w/v glucose solution should be conducted. If *A. gambiae* females were repelled from taking an ATQ-treated sugar meal, they would likely be most deterred from sugar meals with the highest concentration, 100μM ATQ. Under the assumption that, at 100μM, all of the infected mosquitoes were ones that didn't feed on the ATQ-treated sugar meal, the repellency of sugar feeding in individual *A. gambiae* females could be obscuring a significant dose-dependent effect on oocyst intensity at 10μM and 1μM ATQ. The expectation to see a decrease in oocyst intensity with increasing ATQ concentration is due to a similar experiment done using tarsal contact, as previously mentioned. However, this experiment was done with a 6-minute exposure right before an infectious blood meal and through tarsal contact exposure, so the two experiments cannot be directly compared. One just serves as an example of dose-dependent effects from a dilution series assay. It is, nonetheless, logical to assume that oocyst intensity should be affected before the prevalence of infection is affected, given that a decrease in oocyst intensity is seemingly a milder effect than a decrease in the prevalence of infection.

The distinct effects on oocyst intensity could also result from differences in ATQ exposure through tarsal contact versus through sugar feeding. In sugar feeding, the ATQ-treated sugar meals, like most glucose-based sugar meals, will enter into the crop, a second stomach that stores the sugar and releases it over time into the midgut. The highly lipophilic ATQ could potentially be transported to the midgut in two ways: 1) pumped into the midgut with the sugar meal, or 2) diffused through the crop wall into the

hemolymph and eventually into the midgut. Through tarsal contact, ATQ potentially penetrates the cuticles and circulates in the hemolymph, where it will diffuse into the midgut lumen. In sugar feeding experiments, the wide range of exposure (24 hours) makes it difficult to track precisely when *A. gambiae* females are taking sugar meals. The difference in metabolism in an *A. gambiae* female taking her ATQ-treated sugar meal at the beginning of the 24-hour exposure versus at the middle or end of the 24-hour exposure is unclear. This can be clarified by shortening the exposure time. Another consideration is how the sugar meal is affected by the blood meal and vice versa. Since the mosquitoes take a blood meal right after the 24-hour exposure, it is unclear how much of the sugar meal taken before the blood meal will be digested, and thus, it is unknown whether the ATQ effects stem from ATQ circulating in the hemolymph, ATQ entering the midgut with the sugar meal, or both. Nonetheless, ATQ, through sugar feeding, was able to circumvent tarsal contact, which is heavily affected by cuticular resistance in the field.

Previous experiments showed that a 6-minute tarsal contact exposure, 24 hours before a blood meal, completely suppressed the development of oocysts. The same results were seen with a 6-minute tarsal contact exposure right before a blood meal (Paton et al., 2019). This shows that ATQ, through tarsal contact, was still active after 24 hours in the mosquito and was not fully metabolized during that time. It also showed that ATQ, through tarsal contact, has a fast mode of action, being able to inhibit early mosquito-stage parasite development when given immediately before an infectious blood meal. These results suggest that ATQ circulation in the hemolymph is sufficient in blocking

malaria transmission if it were administered any time between 0-24 hours before an infectious blood meal. If ATQ circulation in the hemolymph is the mechanism for tarsal contact assays, it leads to question whether potential ATQ diffusion into the hemolymph during sugar feeding is sufficient for arresting parasite development and, if so, what effects ATQ propelled into the midgut with a sugar meal has on the mosquito and parasite development. Further research must be done to understand the pathway ATQ takes to reach the parasites in the midgut and if timing of the meal has an effect on oocyst intensity and prevalence of infection as well. This could include extracting the hemolymph and midgut and determining the concentration of ATQ found in each compartment at different time points before and after a blood meal. Moreover, a better way to parse out the different effects of tarsal contact and sugar feeding on oocyst prevalence and intensity is to run the infection experiments side-by-side, as the lack of a dose-dependent change in oocyst intensity could be specific to the infections rather than to the treatments.

The transmission of *P. falciparum* is stopped when *A. gambiae* females are exposed to continuous post-infection ATQ-treated sugar meals. The median oocyst intensities per midgut in the treated groups at day 7, day 10, and day 14, were noticeably lower than the median oocyst intensities per midgut in the associated controls, but they were not significant. Only on day 7 is the prevalence of infection of the ATQ-treated group significantly lower than that of its control. Since the mosquitoes at day 7, 10, and 14, all belong to the same group of mosquitoes, they should have a similar prevalence of infection and oocyst intensities. Oocyst development is dependent on the number of

ookinetes that penetrate the midgut epithelium 2 days pIBM. Therefore, there were no expectations of an increase in the prevalence of infection between day 7 and day 10/14. By the time ATQ-treated sugar meals are provided, the ookinetes should have already encysted into the midgut epithelium. It is possible that at day 7, the oocysts are too small to visualize, even at 400x on a compound inverted microscope. Experiments with higher powers for detection should be used. Immunofluorescent microscopy, where the oocysts can be visualized by anti-Pfs25-fluorescent conjugated antibodies, will provide greater labelling contrast than mercurochrome. Nonetheless, the significantly smaller oocysts show that ATQ has a fast mode of action and is effective soon after administration. There was an increase in the mean size of the oocysts per mosquito midgut despite being fed continuous ATQ-treated sugars from 2 days pIBM. In the post-infection exposures, ATQ merely slows down the parasite growth rather than stops it. Pre-infection ATQ exposures, with both sugar feeding and tarsal contact, arrest parasite growth. This indicates that parasite development before midgut invasion and parasite development after midgut invasion do not rely on the same mechanisms or pathways, and parasite development after midgut invasion does not rely as heavily on cytochrome bc_1 function.

The function of ATQ is to inhibit cytochrome bc_1 in the inner mitochondrial membrane. This prevents ATP production and *de novo* pyrimidine synthesis. In both the formation of zygotes and the formation of sporozoites, DNA replication, and thus *de novo* pyrimidine synthesis, is an important process of development. However, it is unclear whether ATP synthesis or DNA replication plays a bigger role in establishing *P. falciparum* infections in *A. gambiae* females. This can be studied by using transgenic *P.*

falciparum parasites that are able to perform *de novo* pyrimidine synthesis without the cytochrome bc_1 complex by inserting a cytoplasmic dihydroorotate dehydrogenase (DHOD) enzyme, an experiment previously studied by Painter, et al., to determine the necessity of *de novo* pyrimidine synthesis in blood-stage malaria parasites (Painter et al., 2007). DHOD is an essential protein for the *de novo* pyrimidine synthesis pathway and functions via an oxidation-reduction relationship with cytochrome bc_1. ATQ will inhibit the mitochondrial DHOD, thus *de novo* pyrimidine synthesis, and ATP synthesis, but *de novo* pyrimidine synthesis will be rescued by the cytoplasmic DHOD. If normal parasite development in these transgenic parasites does not occur with ATQ exposure, then it is clear there is a greater reliance on ATP than on *de novo synthesis*. By administering ATQ-treated sugar before or after an infectious blood meal while using transgenic cytoplasmic DHOD parasites, it is possible to determine the important mechanisms of cytochrome bc_1 at each point of parasite development. The ATQ-treated feeding assays show that ATQ has stronger effects when administered before an infectious blood meal than after an infectious blood meal. It is hypothesized that parasite development before midgut invasion relies more heavily on the functions of cytochrome bc_1 than parasite development after midgut invasion. Further research must be done to understand the mechanism of ATQ's role on parasite development before and after *P. falciparum* invades the midgut epithelium. Understanding ATQ's role in sugar-feeding before and after malaria transmission to the mosquito can improve the understanding of vector control tools and how and when to apply them.

The significantly smaller size of the oocysts in the ATQ-treated groups suggests that the oocysts development, though not arrested, had not developed into mature oocysts to produce sporozoites by day 14, which is supported when looking at day 14 sporozoite counts. There is complete inhibition of *P. falciparum* infectivity at day 14 by ATQ-treated sugars pIBM. Given more time and the removal of the ATQ-treated sugars, there is a chance that oocysts make a fully recovery and become infectious. However, the observation that ATQ slows down the maturation and development of *P. falciparum* is impressive and points towards significant progress because of the way it will impact the vectoral capacity of *A. gambiae* females by decreasing the vector competence. Even if the oocysts were able to mature and to eventually develop sporozoites, the probability that the infected female mosquito is still alive decreases. Especially if ATQ is used in ATSBs, where toxic compounds could be included in the sugar to shorten the lifespan of mosquitoes, the prolonged pathogen incubation period and decreased survivability from the toxic component of ATSBs could greatly reduce the mosquito's capacity to transmit malaria.

HYD has significant insecticidal effects on adult *A. gambiae* females, leading to greater decreases of survival with 100μM HYD. The three controls were selected to determine whether the extreme 100μM HYD effects were due to repellency to a toxic sugar meal, leading to starvation and death, or due to the insecticide itself. The "nothing" control imitated the repellency effect, and mosquitoes in the "nothing" control group were deprived of sugar and water during the 24 hours exposure period. Despite similar survival trends earlier in the experiment, there was statistically significant evidence that

mosquitoes exposed to 100µM HYD through sugar feeding had a greater risk of dying. The difference between the "nothing" control and 100µM HYD survival was obvious when survival in the "nothing" control group plateaued while the survival in the 100µM HYD group plummeted; none survived after day 7 of the survival assay. While *A. gambiae* females in the "nothing" control group did not survive well, the decrease in survival was rescued after they were given un-treated sugar and water. The effects of 100µM HYD is not due to repellency. HYD would be a great asset in the arsenal of insecticides to use in ATSBs since it is toxic against adult *A. gambiae* females. Nonetheless, mosquito preference isn't taken into account in the survival assays, so it is unclear whether a mosquito would prefer attractive sugar baits with HYD inside when they have other sugar source choices. A similar case could be made when assessing the effectiveness of ATQ in sugar baits, as we don't know if there will be a preference for other sources of sugar meals. In order to better understand how mosquitoes interact with ATSBs in the field, it is important to carry out these studies in field-like settings before implementing them worldwide. A better knowledge of the efficacy of ATQ and HYD in ATSBs can also help eliminate the possibility of developing resistance against these compounds. Since HYD has different molecular targets from previously used insecticides, HYD can also contribute to increasing variation in multiple-insecticide formulas.

HYD was selected due to its already seen effects on reducing the prevalence of *P. falciparum* infection through tarsal contact (Paton et al., 2019). Using sugar feeding to test the effects of HYD on *P. falciparum* infection, however, proved to be difficult since

the mosquitoes died before oocysts could be counted at day 7 pIBM. Through the survival assay, it was determined that 10μM HYD should be used due to its incomplete insecticidal effect on *A. gambiae* females after a 24-hour exposure. In the sugar feeding assay, it is seen that HYD can significantly decrease the prevalence of *P. falciparum* infection in *A. gambiae* but has no effect on the oocyst intensity. Once again, an all-or-nothing effect is seen through the prevalence of infection and the intensity of infection. This is consistent with previous data (see above, Figure 5) that observed similar effects of 1mmol/m^2 HYD exposure on *P. falciparum* development in *A. gambiae* through tarsal contact: HYD had a significant effect on the prevalence of infection but none on the oocyst intensity. From a previous study, HYD is seen to have a 100-fold higher EC50 compared to ATQ in asexual *P. falciparum* parasites (Witschel et al., 2012). This could explain the reduced efficacy of the drug, seeing as 10μM of HYD significantly decreases the prevalence of infection from 89.9% in control groups to 73.8% in treated groups, while 10μM of ATQ decreases the prevalence of infection from 74% in control groups to 34.2% in treated groups. The same experimental design was used.

As seen from the survival assay, the greatest decrease in survival at 10μM HYD doesn't occur until 9-10 days after the exposure. It is likely that HYD, at lower concentrations, has a delayed effect on the survival of mosquitoes, and the longer the mosquitoes survived, the more the HYD is metabolized and cleared from the mosquito body, hence the plateauing mosquito survival. The delayed effects and eventual clearance of HYD could also explain why there were no changes in the oocyst intensity. The decrease in prevalence could be due to mosquitoes with slower metabolism. The

mosquitoes with no infections could be mosquitoes that could not fully metabolize HYD and will eventually die from HYD. Since the infection experiment cut off on day 7, the effects of HYD on the oocysts, infection, and mosquito survival might not have been fully visible. It will be interesting to see if there is any effect on oocyst size with HYD treatment or prolong the study to see if there is any effect on the parasite's ability to develop transmissible sporozoites. Additionally, removing mosquitoes at earlier time points—before some mosquitoes die from HYD toxicity—and looking at the oocyst intensity and prevalence of infection could also improve understanding of HYD's effects during malaria infection in mosquitoes. In terms of malaria control, a delayed antimalarial effect could be beneficial when combined with ATQ's fast-acting antimalarial effect by substantially decreasing malaria transmission even after multiple infectious blood meals. Further research must be done, however, to determine whether HYD does have a delayed antimalarial effect when administered through sugar feeding.

MET, an insect growth regulator, is an agonist of 20E, an important ecdysteroid hormone involved in many aspects of an insect life cycle, such as in molting, mating, and egg development. Previous research on the effects of MET on adult *A. gambiae* females was done by topically applying MET on their thoraxes (Childs et al., 2016). However, this is not applicable for the field, as current popular malaria and vector control tools, such as IRS and LLINs, typically target tarsal uptake. Thus, MET was administered through tarsal uptake to determine if the its effects through topical application on the thorax could be replicated. There were no significant changes in oocyst intensity, in prevalence of infection, nor in egg development and eggs laid when comparing

1mmol/m^2 MET to the control. This could suggest that through tarsal contact, insufficient concentrations of MET enter the mosquitoes. The results suggest that MET through tarsal uptake is not an efficient method of administering MET, even with the addition of RME that was shown to enhance the bioavailability of several insecticides. There were also no significant differences in 20E concentration levels when mosquitoes were exposed to RME+MET 0 hours before the blood meal and 24 hours before the blood meal. Childs, *et al*. showed that MET caused significant changes in egg development, ovipositioning, and *Plasmodium* infections when topically applied to the *A. gambiae* thorax (Childs et al., 2016). MET that is applied topically onto the thorax tends to have better absorbance because the acetone mixed into the compound dissolves bits of the cuticle to dissolve larger amounts of MET into the mosquito. On the other hand, tarsal contact plates only have 1 mmol per m^2 of MET, and there is no way to visually the uptake of the compound.

There was, however, a significant decrease in the number of eggs developed per female after *A. gambiae* females were fed MET-treated sugar meals. This suggests that sugar feeding could be an effective way of delivering MET. However, Werling et al., showed that disrupting 20E signaling by knocking down EcR resulted in decreased egg development per mosquito and decreased oocyst intensity. A decrease in oocyst intensity per midgut might not always be a sign of decreased transmission. It has shown that a decreased oocyst intensity per midgut often led to an increased oocyst size and thus faster sporozoite development and transmission of malaria (Werling et al., 2019). MET's disruption of 20E signaling is by binding to EcR, which does not have the same effect as knocking down EcR, but similarly, there was a decrease in the number of eggs developed

per mosquito. Further research needs to be done to assess MET-treated sugar feeding has an effect on the prevalence or intensity of infection and on the oocyst size or parasite transmission. Caution must be taken when disrupting the 20E signaling pathway, as a decrease in egg development and in parasite intensity could lead to faster transmission.

When comparing the three compounds and their potential as vector control compounds, it is clear that ATQ is exceptionally suited for vector control. In cases of tarsal contact and sugar feeding, ATQ was able to significantly halt the transmission of malaria parasites and does not have known toxic effects against the mosquito itself. HYD works relatively well in terms of decreasing the prevalence of infection in *A. gambiae* females and works extremely well as an insecticide, to decrease the survival of *A. gambiae* females after a 24-hour exposure to the 10% glucose solution *ad libitum*. Nonetheless, its efficacy in sugar baits has yet to be tested. Previous studies on HYD in ant baits suggest that HYD might be photolabile, which might impact the stability of HYD in field-like conditions (Hooper-Bùi & Rust, 2001; Van der Meer et al., 1982). MET through tarsal contact has shown no significant effects on infection nor on egg development. However, a significant decrease in the number of eggs developed after exposure to MET-treated sugar meals was observed. However, as a warning from the Werling et al.'s 2019 study, disrupting reproductive pathways in *A. gambiae* might not be the best solution for eliminating malaria transmission (Werling et al., 2019).

A. gambiae female adults will typically have their first blood meal at day 5 after they have become adults. As female mosquitoes grow older, the likelihood of carrying *P. falciparum* increases. *A. gambiae* tend to feed indoors at dusk, and they will likely rest

indoor as well. Many of the current malaria control tools target this indoor feeding and resting behavior. However, not all *Anopheline* mosquitoes that carry *Plasmodium* dwell indoors, with some mosquitoes adapting their behaviors to circumvent the increasing number of insecticides inside homes (Ranson et al., 2011). The significant impact that HYD and, in particular, ATQ has on the prevalence of infection through sugar feeding can be used to target those mosquitoes that feed outdoors or are shifting their behaviors biting and resting behaviors. Even without behavioral resistance, mosquitoes that feed and rest indoors must travel out to find sugar meals, so using sugar baits as a vector and malarial control tool broadens the range of mosquitoes that can be targeted (Qualls et al., 2015). Nonetheless, more research needs to be done on the sugar feeding behavior of mosquitoes to improve the use of ATSBs for malaria and vector control and to decrease the chances of resistance building against these compounds.

Future Directions

Despite the significant effects of ATQ on the development of *P. falciparum* in *A. gambiae*, it is important to fully understand the limitations of ATQ before using it for malarial control. With the oocysts increasing in size despite continuous exposure to ATQ, it is important to assess whether the oocysts will eventually mature and develop sporozoites despite the continuous ATQ exposure. This can be done by extending the study until 21 or more days. Furthermore, it is also important to see the effects on oocyst size and sporozoite transmission when the continuous ATQ is taken away after a few days of feeding. This can be done by removing ATQ-treated sugar exposures and by

replacing them with regular 10% glucose solutions to see if the infection can be rescued by eliminating ATQ. Since there is potential for ATQ-use in ATSBs, exploring the type of toxic compounds, such as HYD, and attractants that ATQ could be mixed with in ATSBs can contribute greatly to malaria control tool development. Historically, malaria control failed due to the lack of combination therapies and vector control tools that targeted multiple points in a pathway or organism. It is important that moving forward, caution is taken to ensure resistance does not build against the tools we are currently developing. It is important to be careful to fully understand the effects of a compound on malaria transmission before placing them in control tools .

To support malaria control tool development, there needs to be more antimalarial and insecticidal compounds that will work on LLINs and in ATSBs as well. The current arsenal of useable compounds is diminishing, with greater insecticide and antimalarial resistance, so more compounds are needed to replace those that no longer work as effectively. On a more molecular level, it will be interesting to understand how much ATQ is actually circulating through the hemolymph of the mosquito after a blood meal or through tarsal contact. This can also explain how other such compounds circulate through the mosquito body when fed through sugar meals. By quantifying the amount of ATQ that is actually in the mosquito, it will open doors to creating more effective tools against *P. falciparum*. Knowing the quantity of ATQ within the mosquito needed to effectively stop transmission is important to formulate more precise concentrations of treated-sugar baits or LLINs. Additionally, knowing the concentration of compounds in mosquitoes after various methods of intake can help researchers make better decisions on which kind

of compounds to use in which kind of tools. Further studies must still be done to assess mosquito sugar preferences, as ATSBs and other forms of sugar baits would generally be ineffective if mosquitoes are choosing to other sources of sugar than the treated ones. As all of the experiments conducted were done in laboratory settings, it is important to test these forms of malaria control in actual field-like settings, where the tools most similar to what could be distributed will be used to assess the effectiveness of these control measures.

Conclusion

There are lessons to be learned from our previous failures in malaria control. Monotherapies of antimalarials and insecticides have led to developing resistance in both *Anopheles* mosquitoes and *Plasmodium* parasites. While these experiments assess only the effects of single compounds on malaria transmission, it is important to be cautious in the use of one single compound in field settings. History has already shown, through GMEP and after the discovery of artemisinin, that using a single insecticide or antimalarial as a panacea for malaria spells trouble for the future generations and builds resistance against the drug. This research is to supplement tool development, and the compounds tested should be used in combination with other compounds that exerts its function on other targets in the organism. The research shines light on the great potential ATQ has in the future of malaria control and also discusses two other compounds—HYD, which wasn't previously used against mosquitoes, and MET—that could potentially be used in regulating both the parasite and the mosquito.

APPENDIX

Treatment 1	Treatment 2	Risk Ratio[1]	*P*	Lower 95% CI	Upper 95% CI
Nothing	Water	1.9382564	0.0034*	1.2490912	3.007657
Nothing	Sugar	2.1646143	0.0007*	1.3947641	3.3593888
Nothing	HYD 1 µM	2.2831948	0.0003*	1.4711198	3.5435446
Nothing	HYD 10 µM	1.5199827	0.0636	0.9780479	2.3622027
Nothing	HYD 100 µM	0.2852471	<.0001*	0.1700874	0.478377
Water	Nothing	0.5159276	0.0034*	0.3324847	0.800582
Water	Sugar	1.1167843	0.6215	0.7204694	1.7311034
Water	HYD 1 µM	1.1779632	0.4642	0.7598938	1.8260412
Water	HYD 10 µM	0.7842011	0.2791	0.5052368	1.2171943

Water	HYD 100 µM	0.1471669	<.0001*	0.0869789	0.249004
Sugar	Nothing	0.4619761	0.0007*	0.2976732	0.7169671
Sugar	Water	0.8954281	0.6215	0.5776662	1.387984
Sugar	HYD 1 µM	1.0547814	0.8115	0.6804746	1.6349821
Sugar	HYD 10 µM	0.7021957	0.1163	0.4522196	1.0903524
Sugar	HYD 100 µM	0.1317773	<.0001*	0.0776935	0.2235099
HYD 1 µM	Nothing	0.4379828	0.0003*	0.2822033	0.6797543
HYD 1 µM	Water	0.8489229	0.4642	0.5476328	1.3159734
HYD 1 µM	Sugar	0.9480638	0.8115	0.6116275	1.4695626
HYD 1 µM	HYD 10 µM	0.6657263	0.071	0.4286505	1.0339227
HYD 1 µM	HYD 100 µM	0.1249333	<.0001*	0.0735462	0.2122249

HYD 10 µM	Nothing	0.6579022	0.0636	0.4233337	1.0224448
HYD 10 µM	Water	1.2751832	0.2791	0.8215615	1.97927
HYD 10 µM	Sugar	1.4241045	0.1163	0.9171347	2.2113149
HYD 10 µM	HYD 1 µM	1.5021189	0.071	0.9671903	2.332903
HYD 10 µM	HYD 100 µM	0.1876647	<.0001*	0.1121542	0.3140145
HYD 100 µM	Nothing	3.5057323	<.0001*	2.0904016	5.8793292
HYD 100 µM	Water	6.7950082	<.0001*	4.0159997	11.497047
HYD 100 µM	Sugar	7.5885581	<.0001*	4.474075	12.871088
HYD 100 µM	HYD 1 µM	8.0042698	<.0001*	4.7119818	13.596898
HYD 100 µM	HYD 10 µM	5.3286526	<.0001*	3.1845664	8.9162966

Table 2 Statistical analysis of the HYD survival assay.

LIST OF JOURNAL ABBREVIATIONS

BMC.....BioMed Central

PLOS.....Public Library of Science

WHO.....World Health Organization

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